The application of Molecular Biology techniques to analyse diversity in *Theileria parva* populations in Zambia

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by

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Abstract

Theileria parva is a complex protozoan parasite causing East Coast fever in Eastern and Central Africa. Vaccination using live parasites is an effective control measure and has been used in Zambia based on locally isolated and introduced *T. parva* stocks. Diversity among *T. parva* populations was investigated in parasites from two Zambian provinces with different disease epidemiologies and control histories. Isolates from the pre-vaccination era, local and exotic stocks used for vaccination, and one recent field isolate were cloned and passaged *in vitro* to study genomic stability over time.

The results of the data from three genome-wide probes indicate a marked homogeneity and stability among the Zambian isolates in contrast to East African isolates. Results from Southern blot profiles and the polymorphic immunodominant molecule (PIM) sequence analysis suggest a common origin for the Zambian isolates from the pre-vaccination era, except for one isolate (Zam5) from Southern Province. This isolate showed characteristics suggesting a buffalo origin. Assays for genotype characterisation were developed using five allelic markers. Multilocus characterisation revealed identical profiles in a recent Zambian isolate from Southern Province and two components of an exotic cocktail vaccine, indicating the escape of one of the vaccine stocks in the field.

Characterisation of *T. parva* field populations by RFLP-PCR assays after immunisation revealed the presence of dominant genotypes from those that had been used for vaccination. Circumstantial evidence for the involvement of one of the exotic vaccine parasites in epidemics in Southern Province is presented and a hypothesis formulated for the rapid spread of this genotype. Analysis of the characterisation data suggested the existence of two groups of *T. parva* parasites of different origin. The classic *T. parva* group, characterised by a dimorphism of the p150, p104 and p32 loci and the absence of a p67 insert and a buffalo-derived group which showed a polymorphism of p150, p104 and p32 and the presence of a p67 insert. There is evidence that recombination occurs, resulting in parasites that have characteristics of both groups. The relevance of these recombinant parasites in the epidemiology of the disease seems low. Characterisation of larger samples from areas of regular buffalo-cattle contact is necessary to clarify this. Sequence analysis of the most discriminative locus (PIM) was undertaken and gene conversion could be the main mechanism generating diversity. A more appropriate nomenclature for *T. parva* is proposed based on the growing evidence of molecular differences among isolates and stocks.

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List of abbrevations

PIM	polymorphic immunodominant protein
ECF	East Coast fever
NK	natural killer cell
CTL	cytotoxic T lymphocyte
pCTL	precursor cytotoxic lymphocyte
TCR	T-cell receptor
MHC-I	major histocompatibility locus I
MHC-II	major histocompatibility locus II
HLA	human leucocyte antigen
HIV	Human immunodeficiency virus
Mr	molecular weight
HD	Heteroduplex
RFLP	Restricted Fragment Length Polymorphism
SSCP	Single Stranded Conformation Polymorphism
Mab	monoclonal antibody
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
cDNA	complementary DNA
LD	linkage disequilibrium
PAGE	Poly Acrylamide Gel Electrophoresis
AA	Adultes (ticks)
NN	nymphes (ticks)
LL	larvae (ticks)
CMI	Cell Mediated Immunity
ITM	Infection and Treatment Method
LD 50	Lethal Dose 50
PBL	Peripheral Blood Lymphocytes
MSI	MacroSchizont Index
Bp	BasePair
EIR	Entomological Inoculation Rate
FAO	Food and Agriculture Organisation
BADC	Belgian Animal Disease Control project
TAS	Telomere Associated Sequences
HR	Homologous Recombination
kDA	KiloDalton

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1. Introduction

1.1. Theileria parva

1.1.1. General

Theileria parva (Theiler, 1904) is a tick-borne protozoan parasite causing a disease complex in cattle known as East Coast fever, Corridor disease and January disease. Its distribution is restricted to the eastern, southern and central parts of Africa, corresponding to the range of its major vector, *Rhipicephalus appendiculatus*. It has a restricted host range and is the most pathogenic species of all *Theileriae*. It causes economic losses over its entire range estimated in 1989 by Mukhebi *et al.* (1992) to be $ 168 million annually.

The latest classification of *T. parva* as revised by Levine *et al.* (1980):

Kingdom **Protista**

Subkingdom Protozoa

Phylum **Apicomplexa** (apical complex present, syngamy)

Class **Sporozoea** (sporozoite forming)

Subclass Gregarinia - Coccidia - **Piroplasmia** (piroform parasites)

Order **Piroplasmida** (asexual/sexual reproduction, ticks as vector)

Suborder

Family **Theileriidae** (schizont stages in mononuclear cells)

Genus ***Theileria*** (piroplasms in erythrocytes, lack pigment)

Species *mutans* *velifera* *taurotragi* ***parva*** *annulata* *buffeli* *sergenti* *lestoquardi* *ovis*

This classification corresponds well with the phylogenetic data obtained from various DNA comparisons (Allsopp *et al.,* 1994). Small sub-unit ribosomal (Ssu rRNA) gene analysis revealed that *T. parva* and *T. annulata* are more closely related to each other than to the other *Theileria* spp. (Allsopp *et al.,* 1994).

Cattle, African buffalo (*Syncerus caffer*) and waterbuck (*Kobus defassa*) are the only known hosts for *T. parva*. The African buffalo is a reservoir of diverse *T. parva* populations from which the bovine parasite is generally considered to have originated (Lohr, 1978; Young, 1981a; Conrad *et al.,* 1989a). *Theileria parva* is apathogenic in buffalo but when transmitted to cattle causes a very severe disease known as Corridor disease. Very few piroplasms are produced in this form of the disease (Young *et al.,* 1973b), so that the disease usually dies out in the absence of buffalo-derived infected ticks (Stagg *et al.,* 1983). This characteristic was used to support the case for consideration of the buffalo parasite as either a separate species, *Theileria lawrencei*, or subspecies, *Theileria parva lawrencei*, although the parasite is antigenically and morphologically very closely related to the *T. parva* parasite of cattle (reviewed by Uilenberg, 1981 and Dolan, 1987). A clear biological basis for this subspecies classification is lacking.

The trinomial classification proposed by Uilenberg (1981) has been abandoned as the molecular genetic characterisation and cross-immunity data did not substantiate subspecies within the *T. parva* complex. It was recommended that the parasite stocks be described as either cattle- or buffalo-derived parasites (Anon. 1989). This classification is accepted but it will not be followed in this discussion in order to allow a clear distinction to be made among cattle-derived *T. parva* and *T. lawrencei*, and buffalo-derived *T. lawrencei* parasites. Cattle-derived parasites will be referred to as *T. parva* and buffalo parasites as *T. lawrencei*. Other *T. lawrencei* parasites will be defined as cattle-derived and buffalo-derived *T. lawrencei* depending on their transmissibility. Scientific evidence will be presented later in favour of a classification that recognises distinctions between *T. lawrencei* and buffalo-derived *T. parva* parasites.

The parasites normally cycling in cattle are referred to as *T. parva*. This system of nomenclature would also define *T. parva* parasites cycling in buffalo as *T. parva*. The role of buffalo-derived parasites in *T. parva* strain dynamics is still unclear, although it is believed that they could play a role in the generation of novel *T. parva* strains (Lohr, 1978). Transmission of *T. lawrencei* from buffalo to cattle occurs regularly but the establishment of a buffalo *T. lawrencei* parasite in cattle depends on the ability of the parasite to produce blood stages and be infective to ticks. The process of change from a buffalo-adapted parasite inducing low parasitaemia to a cattle-adapted type, producing abundant schizonts and piroplasms, is called 'transformation'. This is thought to involve a gradual adaptation of the parasite over several cattle-tick passages whereby the intralymphocytic and blood stages of the parasite increase in abundance in the cattle host. The result is that a buffalo-derived parasite becomes morphologically and behaviourally identical to a cattle *T. parva* parasite. The mechanism of this transformation is not known. It could consist of a selection of a subpopulation(s) of the infecting parasites or a true adaptation of some biochemical pathway or biological system of the parasite to the cattle host. For the sake of clarity and discussion, and in view of increasing molecular evidence, it is felt that the name *T. lawrencei* would better define the original *Theileria* species in buffalo. Subpopulations of *T. lawrencei* might have evolved which adapted (transformed) to cattle and induced classical *T. parva* or cattle-derived *T. lawrencei*. Buffalo-derived *T. lawrencei* would define those buffalo-derived parasites that infect cattle occasionally but would die out and be unlikely to cause large epidemics (such as Corridor disease).

Various authors have reported successful transmission of *T. lawrencei* from buffalo to cattle. Subsequent passages in cattle have been moderately (50%) successful, but transformation was reported to be successful only with *T. lawrencei* parasites from Kenya and Tanzania. Attempts have also been made to alter the disease course of buffalo-derived *T. lawrencei* through serial passage in cattle. No transformation occurred with isolates from South Africa (e.g. Hluhluwe isolate, De Vos, 1982) or from Zimbabwe (Lawrence, 1981) after five passages. One explanation for this may be that the South African transformation

attempts were made using *R. appendiculatus* from Kenya. These ticks might not have been suitable vectors for the southern African buffalo-derived *T. lawrencei* stocks. Tick transmission experiments with the closely related *R. zambesiensis* were successful and have given much higher infection rates with the same buffalo-derived *T. lawrencei* stock (Blouin and Stoltsz, 1989). *Rhipicephalus zambesiensis* ticks are drier area ticks, replacing *R. appendiculatus* in the southern African region (Walker *et al.,* 1981b; Lawrence *et al.,* 1983). Another important factor may be the epidemiological differences between eastern, central and southern Africa, which influences the frequency of transmission and the chances of transformation. In view of recent data, which seems to contradict the accepted view of the buffalo-based reservoir for new *T. parva* strains, a more detailed review of the relevant literature will be presented.

Three documented attempts, involving four to six cattle passages with buffalo-derived isolates from Kenya or Tanzania, resulted in transformation. In at least one attempt, the presence of a concurrent *T. parva* infection seems very likely, based on parasite characterisation data.

Barnett and Brocklesby (1966) transmitted a Kenyan buffalo isolate to cattle and the piroplasm parasitaemia was shown to increase markedly from the 4th passage onward.

The transmission of a parasite from a buffalo from the Serengeti National park, Tanzania, to cattle was quantified by Young and Purnell (1973). They achieved transmission in six out of eight tick feeds from two young buffalos to cattle. Transmission between cattle was more successful in the second passage and parasitaemia increased markedly after the fourth passage, but no detailed *in vitro* characterisation was done (Young and Purnell, 1973).

In 1987, Grootenhuis *et al.* (1987) used the same buffalo-derived *T. parva* transformed isolate from Serengeti as used by Young and Purnell, (1973) to characterise stabilates derived after bovine passages three and six. All cattle became lethally infected with a *T. parva* Muguga-like parasite whereas buffalos showed a mild transient infection without the usual carrier state.

They obtained a *T. parva* Muguga-like monoclonal antibody (mAb) profile in cattle, but variable buffalo related mAb profiles in buffalo. There was not complete cross-protection between *T. lawrencei* and *T. parva* 'transformed' parasites in buffalo (Grootenhuis *et al.,* 1987). The authors concluded that the initial stabilate (from passage three) contained a *T. parva* Muguga-like parasite. This assumes that either buffalo could harbor parasites, that can be enriched or be transformed to a *T. parva* Muguga-like parasite after several bovine passages, or that a superinfection with the *T. parva* Muguga type had occurred. The latter is more likely in view of the results obtained with mAb profiles and cross-protection. This raises the possibility of contamination with a *T. parva* Muguga type parasite during the pick-up of the second, and only successful passage, in 1973. The *T. parva* Serengeti-transformed component of the Muguga cocktail stabilate (FAO Malawi stabilate 69, first passage) that is used for immunisation in a number of countries in the region has been derived from this stabilate (Radley *et al.,* 1975b). Recent molecular characterisation of this component reveals marked similarity to *T. parva* Muguga, supporting the likelihood of contamination. These issues raise questions about the nature of the *T. lawrencei* parasites and the justification for the inclusion of the *T. parva* Serengeti transformed component in the Muguga cocktail vaccine.

Maritim *et al.* (1992) were successful in transforming a parasite, using a stabilate made from ticks fed on a buffalo (nr.7014) captured on Ol Pejeta ranch in Laikipia District, Kenya (Mutugi, 1988). Tick passage was initiated by feeding ticks on a bovine carrier immunised with the above buffalo-derived stabilate. Complete cross-protection was shown between the original and the fourth passage parasite but a marked increase in parasitaemia was only recorded after the fourth passage, indicating transformation to a *T. parva* like parasite. The mAbs characterisation profile did not change significantly over the six passages, although mAb20 (buffalo specific mAb) was not present in the fourth passage. The relevance of this is unknown, but it is the only transformation experiment supported by reasonable characterisation data. This study provides consistent evidence for a transformation event and the possibilities of using the transformed parasites in *T. parva* vaccines as demonstrated by the complete

cross-protection achieved between the transformed and the *T. lawrencei* stock. It is disappointing that DNA characterisation methods were not used, and the isolates are not available for retrospective studies.

When cattle survive a *T. lawrencei* infection, they usually become carriers (Barnett and Brocklesby, 1966; Dolan, 1986a and b; Potgieter *et al.,* 1988). The carrier populations were shown to be immunological different from the parent stock when challenged with the parasites of the primary infection (Maritim *et al.,* 1989). The conclusion was that the carrier state is probably due to the persistence of a subpopulation from the original mixture of immunogenic components in the stabilate. Therefore, the authors argued that immunisation using *T. lawrencei* stabilates would not cause a threat to cattle by producing new variants. However, an alternative interpretation could be based on the appearance of new antigenic types from recombination. The carrier stabilate was produced from a primary *T. lawrencei* stabilate (the same as that used by Maritim in 1992) after two tick passages in cattle. This could have created opportunities for recombination among different subpopulations, in the primary stock. Besides this, the immunisation methods used for the carrier animals were not identical. Different tick-induced infections were used and this could explain some of the discrepancies in their results. The mAbs used to characterise the different carrier populations would not reveal most recombinants as the majority has been shown to react with epitopes present in the protein coded by one gene (Toye *et al.,* 1991). It is known also that protection is not always reciprocal as *T. parva* stocks protecting against challenge with another stock might not protect when the immunization and challenge roles are reversed (Irvin *et al.,* 1983).

The conclusion from these transformation studies suggests a reservoir of *T. parva* in buffalo and the possibility that cattle-adapted *T. parva* could circulate between buffalo and cattle in endemic areas in Kenya and Tanzania. This implies the possibility of the generation of new antigenic types although it does not provide unequivocal proof for this. Furthermore, it could be argued that these data support the possible occurrence of recombinants between *T. lawrencei* and a contaminating *T. parva* (Muguga) with the generation of new antigenic types. It

has been shown that buffalo can be infected by *T. parva* parasites and subsequently produce infected ticks (Walker, 1932; Lewis, 1943 and Brocklesby, 1964) and become carriers (Barnett and Brocklesby, 1966; Brocklesby and Barnett, 1966). There is no reason to doubt that this could also be happening in the field under endemic conditions in Kenya or Tanzania.

Additional evidence in support of the rarity of transformation comes from molecular data. The use of probes as molecular characterisation tools has revealed a large diversity among *T. lawrencei* stocks (Conrad *et al.,* 1989a; Bishop *et al.,* 1994). In a survey in Zimbabwe, different Tpr1 genotypes were found in almost every buffalo-derived isolate, whereas a predominant Tpr1 genotype was found in bovine isolates (Bishop *et al.,* 1994). Sequence data of p67, a sporozoite stage-specific surface antigen of the parasite show a single, highly conserved allele, even at the nucleotide level, in all *T. parva* stocks (Nene *et al.,* 1996). In contrast, variable alleles are found in p67-genes of buffalo parasites, with a 80 bp insert in the majority of stocks (Nene *et al.,* 1996; Collins, 1997). Thus there is growing evidence that only a limited number of *T. lawrencei* strains might become established in cattle after transformation or selection. Therefore, the exact role of *T. lawrencei* in the epidemiology and control of ECF is still not full elucidated. This has important repercussions as it has been demonstrated that it is difficult to protect *T. parva* immunised cattle against *T. lawrencei* (Radley *et al.,* 1979) and incomplete cross-protection has been demonstrated between *T. parva* and *T. lawrencei* (Young *et al.,* 1973c). With the development of molecular characterisation techniques, the role and risks of buffalo parasites can be examined with a view to defining the problems they might cause and helping to develop optimal strategies for immunization.

1.1.2. Distribution range.

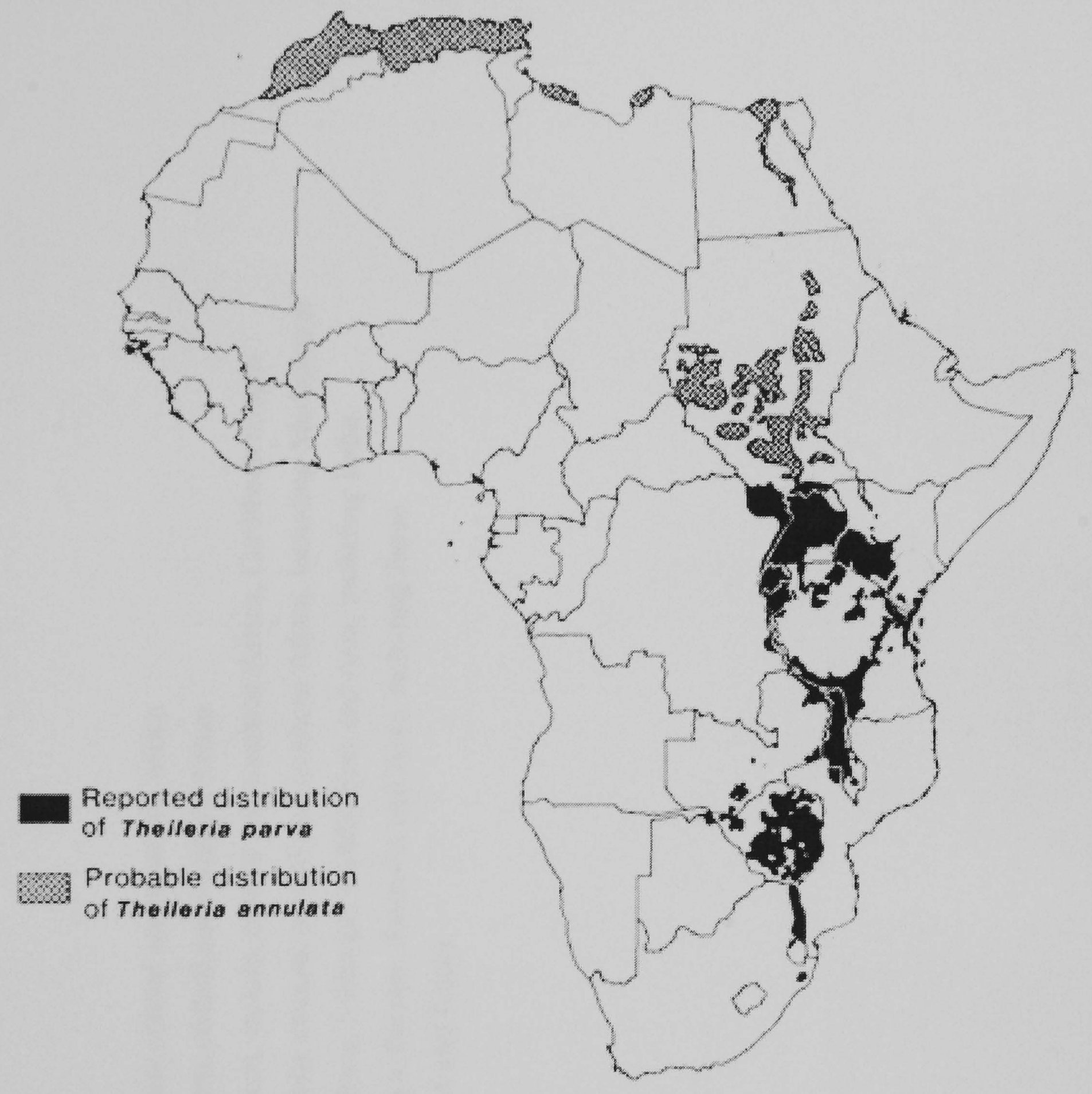
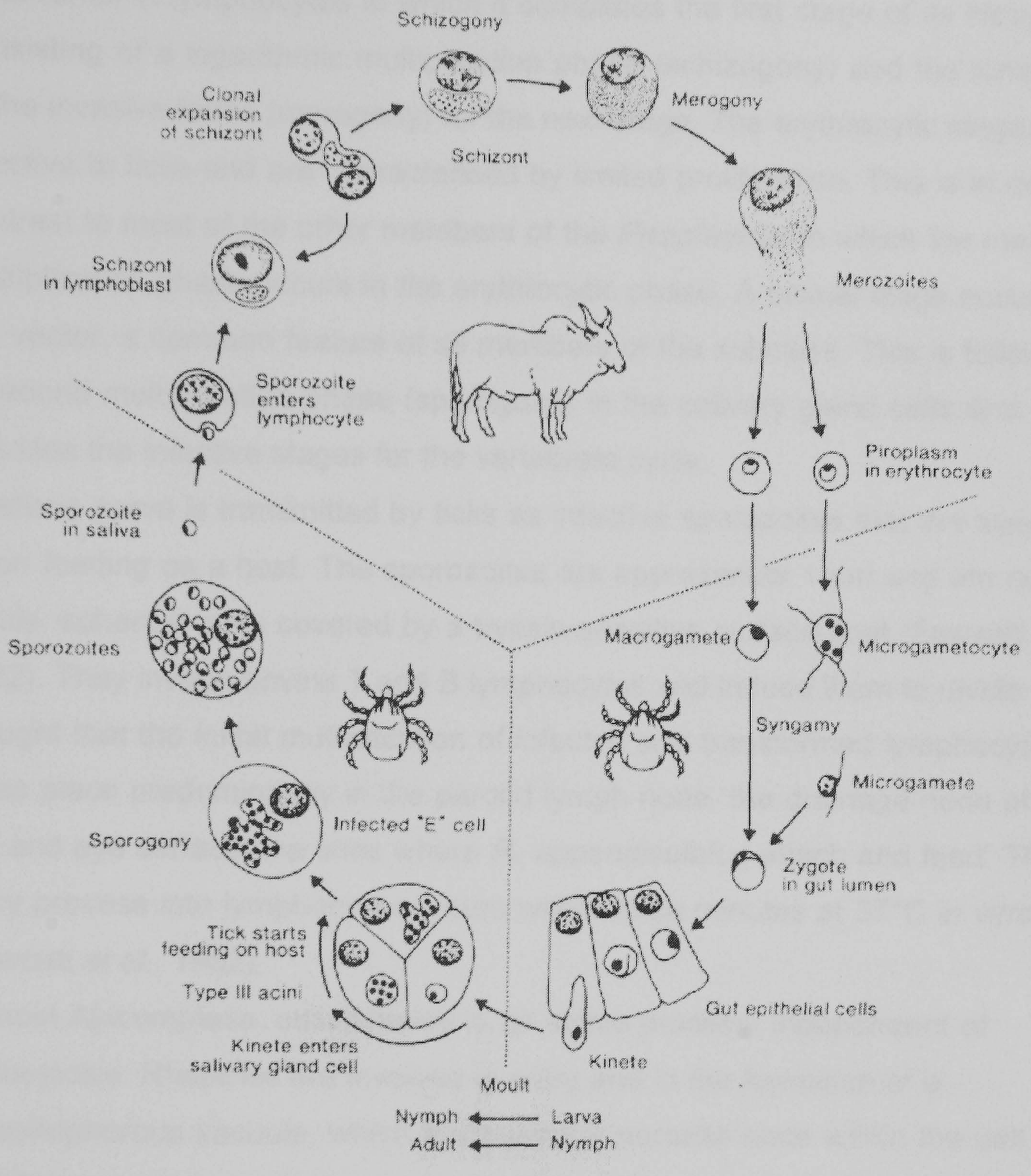


Fig.1 Distribution range of *T. parva* and *T. annulata* in Africa. Picture from Norval, R.A.I.; Perry, B.D. and Young, A.S. (1992). The epidemiology of Theileriosis in Africa. Academic Press, London.

Parasite	Vector	Distribution
T. parva	*Rhipicephalus* spp	eastern, central and southern Africa
T. taurotragi	*Rhipicephalus* spp	eastern, central and southern Africa
T. mutans	*Amblyomma* spp	western, eastern, central and southern Africa, Caribbean islands
T. velifera	*Amblyomma* spp	western, eastern, central and southern Africa, including Madagascar
T. annulata	*Hyalomma* spp	N.Africa, S.Europe, Middle East and Asia, including India
T. buffeli	*Haemaphysalis* spp	Kenya, Europe, Australia and Asia, including Japan
T. sergenti	*Haemaphysalis* spp	Japan and Korea

1.1.3. Life cycle



Life cycle of *Theileria parva* in cattle and in the ixodid tick *Rhipicephalus appendiculatus*. Figure prepared by A.S. Young.

Fig. 2 Life cycle of *Theileria parva*. Picture from Norval, R.A.I.; Perry, B.D. and Young, A.S. (1992). The epidemiology of Theileriosis in Africa. Academic Press, London.

The life cycle of *T. parva* is complex. It involves the sequential invasion of two different cell types in the cattle host and a sexual cycle in the vector. The parasite establishes in lymphocytes in which it completes the first stage of its lifecycle consisting of a logarithmic multiplication phase (schizogony) and the formation of the invasive forms (merogony) for the next stage. The erythrocytic stages are infective to ticks and are characterised by limited proliferation. This is in marked contrast to most of the other members of the *Piroplasmia* in which the main multiplication phase occurs in the erythrocytic phase. A sexual stage occurs in the vector, a common feature of all members of the subclass. This is followed by a second multiplication phase (sporogony) in the salivary gland cells and provides the infective stages for the vertebrate cycle.

Theileria parva is transmitted by ticks as infective sporozoites that are injected when feeding on a host. The sporozoites are approximate 1 µm and are non-motile, spherical cells covered by a trypsin-sensitive surface coat (Fawcett *et al.,* 1982). They invade bovine T and B lymphocytes and induce them to divide. It is thought that the initial multiplication of infected and transformed lymphocytes takes place predominantly in the parotid lymph node, the drainage node of the ear and eye surface, the sites where *R. appendiculatus* attach and feed. The entry process into lymphocytes occurs within three minutes at 37°C *in vitro* (Fawcett *et al.,* 1982).

In most Apicomplexa, cell invasion is an active process, independent of endocytosis. Rhoptries are involved in entry and in the formation of a parasitophorous vacuole, which encloses the parasite once within the cell. Due to differences in the apical complex of *Theileria* species, in which only rhoptries and spherical bodies represent the apical complex (Shaw *et al.,* 1994), the cell invasion process, and consequently the intracellular location, differs. The binding of the sporozoite to the lymphocyte is a passive ligand-receptor mediated process. The invasion is a passive endocytotic mechanism described as progressive circumferential zippering (Fawcett *et al.,* 1982). During entry, most of the sporozoite surface coat is lost (Webster *et al.,* 1985). It has been shown that the presence of the sporozoite surface coat is required to achieve the entry into the lymphocytes (Musoke *et al.,* 1982 and 1984; Toye *et al.,* 1995). The

role of host cell or parasite surface proteases in the entry process is not clear, nor has the lymphocyte receptor(s) for the sporozoites been identified.

The contents of the rhoptries are discharged soon after entry which coincides with the dissolution of the host cell membrane. Therefore it appears that the rhoptry content participates in the dissolution of the host cell membrane. The parasite at this stage lies free in the host cell cytoplasm and becomes associated with the host cell microtubules (Stagg *et al.,* 1980; Shaw *et al.,* 1991). Two other sets of secretory organelles known as micronemes and microspheres (dense granules) are located in the parasite cytosol and are thought to play an important role in the establishment of the parasite in the host cell (reviewed by Shaw, 1997 and Sam-Yellowe, 1996). The infected cell undergoes major changes and is induced to divide by the developing schizont stage of the parasite. The schizont of *T. parva* becomes associated with the mitotic spindle (Hulliger *et al.,* 1964) of the lymphocyte and divides in synchrony with the host cell, being pulled apart as the daughter cells separate. This gives rise to a clonal expansion of the initial sporozoite infected cells. This transformation enables this stage to grow as a continuous line in *in vitro* cell cultures. The parasite stimulates cytokine production (reviewed by McKeever *et al.,* 1997) which drives this growth. The transformation is reversible, treatment with specific antitheilerial drugs eliminates the parasite and the host cell stops dividing. Thus the proliferation is parasite dependent (Pinder *et al.,* 1981). The process of parasite-induced transformation is not understood, but some of the molecular mechanisms involved in maintaining this transformation are known (Eichhorn and Dobbelaere, 1994; Carrington *et al.,* 1995, Demartini and Baldwin, 1991). It is thought that T cells recognize a surface determinant on the infected cell and, together with a second signal, probably provided by exogenous IL-2, induces their proliferation (Collins *et al.,* 1996).

One of the first changes in the host after infection is a marked increase in the lymphoblast population, making up 25% of all lymphocytes in the drainage lymph node (Emery, 1981b). This is accompanied by an increased expression of various cytokines, creating a favourable environment for rapid multiplication of the parasite (McKeever *et al.,* 1997). Haematogeneous dissemination of infected

lymphocytes occurs within the first 2-3 days after inoculation (Emery, 1981b; Shatry *et al.,* 1981). After 5-6 days, depending on the numbers of sporozoites injected, the first multi-nucleate schizonts are seen in the drainage lymph node followed 3-4 days later in the rest of the lymphoid system (Shatry *et al.,* 1981). Patency, the detection of schizont-infected lymphocytes in the lymph node is usually between seven and nine days following infection, corresponding with 1% of lymphocytes infected. The growth is not entirely exponential (Morrison *et al.,* 1981) and the infected cells invade other lymph nodes and various tissues, causing a generalised pathology, which is often lethal.

A proportion of schizonts will differentiate into merozoites, the next invasive stage. Merogony starts with the formation of a syncytium through fast division of different nuclei of the schizont. Cell division of the syncytium results in the individualisation of merozoites and host cell rupture. The merozoites possess rhoptry organelles that participate in the invasion of erythrocytes (Shaw and Tilney, 1992) where they differentiate into piroplasm blood stages which are taken up by engorging ticks. Little is known about the factors influencing the induction of merogony or the mechanisms driving the invasion of erythrocytes. No end products of hemozoin have been found in *T. parva*-infected erythrocytes. *Theileria* piroplasms use a cytostome to feed and sometimes a food vacuole is discernible, as in *Plasmodium* (Rudzinska and Trager, 1957 and 1959; Fletcher and Maegraith, 1962; Cox and Vickerman, 1963). This contrasts with *Babesia* which use pseudofood vacuoles (embracing inner cytoplasm) and special coiled organelles for extraparasite digestion of haemoglobin. It is assumed that multiplication of piroplasms does not play an important role in the dynamics of this parasite stage, but schizogony with the formation of four merozoites (maltese-cross forms) has been observed *in vitro* and *in vivo* (Conrad *et al.,* 1986; Fawcett *et al.,* 1987). There is no indication of binary fission (Conrad *et al.,* 1986).

Sexual reproduction in *T. parva* takes place in the tick vector (Gonder, 1910; Mehlhorn and Schein, 1984), which may give rise to recombination between different genotypes (Morzaria *et al.,* 1992b). The piroplasms differentiate into sexual stages inside the erythrocyte after being ingested by the ticks, and are released in the tick gut as the erythrocytes are lysed (Gonder, 1911; Schein *et al.,*

1977). Syngamy follows between micro- and macro-gamete to form a zygote. Large ovoid zygotes of 6-7 µm with microsphere structures are seen within tick gut cells, often in clusters (Mehlhorn and Schein, 1984).

There is still no general agreement about the time of occurrence of meiosis in *T. parva*. Evidence from a fluorescence based DNA technique suggests that the meiotic reduction division occurs during the zygote stage (Gauer *et al.,* 1995), as in *Plasmodium* (Sinden, 1983). Zygotes are frequently found in clusters in tick gut cells, suggesting that they are more likely to be products of meiotic division than of multiple infections (Schein *et al.,* 1977). The ploidy characterisation study described the diploid zygotes differentiating into kinetes in the tick gut epithelium through a two-step meiosis (identical to meiosis in higher eukaryotes) starting with a tetraploid DNA content followed by reduction to a haploid level in a single transition. It has been suggested that this two step meiosis serves as an extra amplification mechanism (Gauer *et al.,* 1995). This is in contrast to *Babesia bigemina* in which a one step meiosis precedes haploid kinete formation (Mackenstedt *et al.,* 1995)

The next stage is a very fast differentiation towards one kinete from a nuclear protrusion into the vacuole of the zygote (this differs from *Haemosporidiae* in which an elongation mechanism forms the kinete). The kinete has a micropore, but no rhoptries as in *Haemosporidiae* or no conoid as in *Plasmodium*.

In engorged nymphae, the first differentiated *T. parva* kinetes are seen from day 12 after repletion and just before moulting. It is not until after the moult (day 16) that motile kinetes are detected in the haemolymph. This is a much slower development than that seen in *T. annulata* infections in which kinetes are found from day 9-10 (Mehlhorn *et al.,* 1978), although nymphal *Hyalomma* ticks moult after 21 days. Shorter periods were found in larvae and have been induced by holding ticks at higher temperatures, which lead to the hypothesis formulated by Young *et al.* (1980a) of a tick ecdysis driven differentiation.

The kinete stage penetrates the tick salivary gland acini to form a sporoblast after the moult (first sporonts at day 20) and it continues its development after the start of a new bloodmeal by the tick. Although some development will occur

when the environmental temperature is elevated to 37°C for several days (Young *et al.,* 1979). Sporogony might be under control of a heat shock gene.

The parasite infects "e" cells of type III acini, which gives them a greater likelihood of being injected into the host, as these cells have a strong excretory function in pumping out excess fluid during blood sucking. They alter dramatically due to rapid increase in fluid secretion (Fawcett *et al.,* 1981). One infected acinus produces 40-50,000 ovoid non-motile sporozoites of ±1µm diameter.

All parasite stages are haploid, except a short diploid zygote stage in the tick intestinal wall (Mehlhorn *et al.,* 1978, Shaw and Young, 1994) as discussed above. As kinetes are haploid and one kinete infects one acinar cell, and in the field, often only one acinus of the salivary gland is found to be infected, it is probable that the sporozoites are products of a single meiotic division and genetically identical (Morzaria *et al.,* 1992).

1.1.4. Clinical disease

Sporozoites are inoculated into the bovine host between one to three days after tick attachment. Disease is characterised in its initial stages by swelling of one of the parotid lymph nodes, the node draining the ear and eye surface which are the predeliction feeding sites of the tick vector. Infected cells will usually appear in the lymph node between 4-9 days after injection of the sporozoites. The incubation period is between 7 and 10 days following natural infection with fever (39.5°C or higher) from about day 10. Fever is a consistent feature. In an acute infection, schizont infected cells disseminate to all lymph nodes, secondary lymph nodes and other organs. Schizonts will be found in the interstitial tissues of the lungs, the gastro-intestinal tract, the kidneys and most other tissues. In the terminal stages of the disease, the temperature may fall below normal, severe pulmonary oedema may develop at the same time of a generalised non-specific lymphocytolysis, suggestive of severe immunopathological events (Visser *et al.,* 1995), thereby often causing death of the host. Maxie *et al.* (1982) described the occurrence of disseminated intravascular coagulation in *T. parva* infections and

suggested this as an important contributing factor in the overall pathology. Panleukopenia develops due to severe suppression of the hemopoiesis (Wilde, 1966; Maxie *et al.*, 1982), and is associated with a progressive lymphocytolysis most likely effected by polyspecific cytotoxic T lymphocytes (CTLs) found during the terminal phase of a lethal infection (Emery and Morrison, 1981c). Pulmonary edema may be closely related to lymphocytolysis of infected cells within the lungs and might be due to activation of complement giving C3a and C5a products with increase of the vasoactive amines affecting cell membrane permeability (Shitaka *et al.*, 1983).

Around the 12th day after infection, merogony takes place in a percentage of schizonts and piroplasms appear in the erythrocytes. Anemia usually develops but its severity is variable depending mainly on the stocks used and is not correlated with the parasitaemia which usually ranges between 1% and 80% (Hill and Matson, 1970; Irvin and Morrison, 1987a). It is known that various cytokine induced molecules such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and nitric oxide (NO) suppress hematopoiesis (Mohan and Stevenson, 1998), and the more severe anemia seen in milder strains could be a secondary effect of a good protective response. Suppression of hematopoiesis alone could not be responsible for this rapidly progressive anemia, and either a non-parasitaemic related erythrocyte destruction or one or another sort of compartimentation would play a role in the cause of this rapid fall in haematocrit. Haematocrit increases and leucocyte responses during recovery are also spectacular and indicative of cytokine influence (Mohan and Stevenson, 1998).

Milder forms of the disease have been reported from the southern edge of the *T. parva* range, characterised by low parasitaemias (max 5%) (Matson, 1967; Lawrence, 1997) and, in the case of Zambian isolates, correlated with marked anemia (Geysen and Brandt, own observations). Anemia and terminal leucocytosis were also reported by Hill and Matson (1970) in their study on January disease (Rhodesian Theileriosis). These parasites have been classified in the past as a *T. parva bovis* subspecies (reviewed by Uilenberg, 1981). There is still no consensus as to the occurrence of milder *T. parva* stocks

in the field as virulence is not a constant property but likely the resultant of the prevailing epidemiological situation. The virulence of *T. parva* stocks and its dynamics will be dealt with under section 1.1.7. and the occurrence of mild strains will be discussed *in extentio* under 1.1.8.

Data are being generated that point increasingly to a key role for cytokines in the pathology of *Theileria* infections. In *T. annulata* there is evidence that the $CD4^+$ T cell arm of the primary immune reaction is altered by the presence of infected cells. Instead of engaging in helper functions with the antigen presenting cells, the majority of the $CD4^+$ T cells were found to be non-specifically activated, with consequent changes in their cytokine profiles. The high IFN-γ production could enhance the growth of parasite infected cells and contribute to the failure of antibody responses. Down regulation of CD-2 adhesion molecules on CTLs abrogates their capacity to lyse infected cells (Campbell *et al.,* 1997).
It has been suggested, from evidence of IL-10 cytokine production in *in vitro* cultures of various *T. parva* infected-lymphoblast populations (McKeever *et al.,* 1997), that a Th2 driven immune response might jeoparadise the normal protective response. There is evidence that IL-10 may interfere with normal $CD4^+$ and $CD8^+$ T cell responses (Brown *et al.,* 1994). It has also been suggested that important increases in IFN-γ in *T. annulata* and *T. parva* are produced towards the end of the infection (Brown *et al.,* 1995). TNF-α production was found, to a lesser extent, towards the end of *T. annulata* infections, but not in the *T. parva* Muguga cell line studied (Brown *et al.,* 1995). These induce upregulation of lymphoproliferation, T-cell mediated cytotoxicity and increased expression of MCH-II antigens contributing to a rapid progress towards lethal disease.

The study of the kinetics of *T. parva* infections has played an important role in understanding the dynamics of the parasite and its underlying pathogenesis (Jarrett *et al.,* 1969). The parasite follows a more or less logaritmic growth curve (see Morrison *et al.,* 1981) from the start of infection, leading to fever (39.5°C) above a threshold macroschizont index (MSI) of 1%. This occurs at a timepoint, determined by the number of sporozoites injected (Dolan *et al.,* 1984b). Piroplasms are detected around day 12, but here the timing appears to be

independant of dose. Virulence might be the result of differences in parasite multiplication times, as slower growing parasites would take longer to produce equal numbers of infected cells. The virulent nature of buffalo-derived infections, characterised by low parasitosis and parasitaemia is probably due to a lack of adaptation of the immune responses of the bovine host to these parasites.

After approximately another 6 days, the MSI may reach nearly 100% and severe lymphocytolysis follows. A proportion of infected lymphocytes will undergo merogony, and the merozoites invade red blood cells where they have been recorded mostly as single piroplasms. However doublets, triplets and rarely quadruplets are also present. They could be the product of multiplication of piroplasms or multiple entry of merozoites. Conrad *et al.* (1986) and Fawcett *et al*. (1987) reported *T. parva* schizogony in red blood cells *in vitro* and *in vivo*, respectively. Intra-erythrocytic multiplication is still not proven in *T. parva*, although if it does occur it does not occur in the high rates seen in other *Theileria* spp. Evidence for multiplication comes from studies using *T. parva* Katete (Zambia), with which it was found that the piroplasm parasitaemia does not follow a Poisson frequency distribution (Delespaux, 1996), This is indicative of a very low rate of intra erythrocytic multiplication. It might be that other mechanisms are affecting distribution, such as compartimentalisation of infected erythrocytes due to altered biochemical properties or selective invasion of cells which, as found in *Plasmodia* and *Babesia*, might play a role also in *Theileria*. Although no experimental evidence has been found, similar mechanisms affecting the distribution of *T. parva* infected erythrocytes in cattle could not be ruled out (Delespaux, 1996).

The low rate found in *T. parva* could be the result of a low multiplication in parasites contributing to the carrier state or more likely the result of limited multiplication during gametogenesis, with developing gametocytes. The former hypothesis can be refuted by the observation that inoculation of blood containing piroplasm does not transmit the disease (Theiler and Du Toit, 1929; Neitz, 1948).

1.1.5. Immune responses

Animals recovered from ECF have a long lasting immunity, giving complete protection against homologous parasite stock challenge and many cross-protect against a number of different, but not all parasite isolates. In challenge experiments, especially when using massive doses (multiple lethal doses), transient but significant numbers of schizonts can often be seen, but immune responses will slow down multiplication and prevent pathology. This protection declines over time in the absence of challenge, indicating that the immunity induced is partially protective, not preventing infection but directed against the schizont stage (Morrison *et al.,* 1995a).

Several experiments have demonstrated that immunity against *T. parva* is cell-mediated and primarily based on CTL responses mounted against the parasite-infected lymphoblast stage (Morrison and Goddeeris, 1990; McKeever *et al.,* 1994; Morrison *et al.,* 1995b), but the exact mechanisms are not known (reviewed by Morrison *et al.,* 1989). Both antigen-specific and non-specific cytotoxic cells have been demonstrated in infected animals. Although most immune cattle mount strong responses to the parasite, only non-specific CTLs are found during the latter stages of lethal infections with no indication of the presence of specific CTLs during primary infections. Moreover, in young calves, CTL responses are minimal during challenge while in adults approximately 30% did not show significant CTL responses in some experiments, although the animals displayed good immunity (Goddeeris *et al.,* 1986). This initiated research towards the role of other cell-mediated immune mechanisms, although no evidence for this was found during a systematic short study by Van de Putte (1992). It was found that cells transformed by *T. parva* were resistant to NK-mediated (natural killer cells) lysis and LAK (lymphocyte activated killing) activity was too variable to be considered as the main protective mechanism (Van de Putte, 1992). Improved knowledge of cell-mediated immunity (CMI) responses revealed the complex nature of CTL-induced responses.

The search for antibodies to parasite-specific antigen on the surface of infected cells (Morrison and Goddeeris, 1990) has been unsuccessful and the identification of parasite-induced antigens, recognised by CTL is currently being

attempted (McKeever and Morrison, 1998). However there is also evidence of non-specific antibody-unrelated antiparasitic effects of sera from severe reactors or recovering animals (Dolan *et al.,* 1985). The mediators of this intralymphocyte killing of parasites have not been identified. The different mechanisms of cell-mediated responses in *T. parva* infections need to be determined. Protective immune responses against intra-cellular protozoa are usually mediated by $CD4^+$ T cell responses (Th1 cells), driven by early IL-12 and IL-2 and low IFN-γ production (Mosmann and Coffman, 1987). Immunity against these complex parasites has been defined as the outcome of the interplay between the subsets of $CD4^+$T cells, their associated cytokines and various effector cells. The fact that *T. parva* infects immune effector cells, which might outgrow the initial T cell population, adds to the complexity of the responses. But the study of the relevance of parasite-mediated phenotypic alterations of infected cells has been difficult. Baldwin and Teale (1987) showed that *T. parva*-transformed cell lines retained their cytolytic activity and antigen specificity, and there are indications that IL-10 expression of *T. parva* infected cells is associated with pathogenesis (McKeever *et al.,* 1997).

From studies of the immune responses in *Plasmodium* infections, it became clear that several immune effector mechanisms can be activated, although the selection criteria are not yet understood (Hill *et al.,* 1997).

1.1.5.1. Humoral responses

Animals also respond to infection with *T. parva* by mounting humoral responses that decline over months in the absence of challenge. Humoral responses towards sporozoite antigens, such as the sporozoite surface coat protein p67 (Musoke *et al.,* 1982) and to a lesser extend the microsphere secreted protein referred to as the polymorphic immunodominant molecule PIM (Toye *et al.,* 1995), have been studied in detail. Neutralizing antibodies against sporozoites are correlated with some protection, although they are not a principal protective mechanism. Piroplasm or macroschizont infected cells give rise to IgG antibodies whereas sporozoites induce IgM, with weak neutralising capacity (Dobbelaere *et al.,* 1984). After repeated challenge with a sporozoite stabilate,

antibodies of the IgG_2 isotype gave good neutralization and blocking of sporozoite invasion of lymphocytes *in vitro* (Musoke *et al.*, 1982).

Humoral immune responses are also directed against PIM, identified on *T. parva* macroschizont infected cells but also transcribed in the sporozoite stage (Toye *et al.*, 1991; Sugimoto *et al.*, 1992 and Kishima *et al.*, 1995). *In vitro* studies have shown that these can also block sporozoite invasion of lymphocytes. These results suggest that both p67 and PIM are involved in the entry process of sporozoites into lymphocytes although an indirect action through steric hindrance of ligands by one of them could not be ruled out (Toye *et al.*, 1995).

This observation, and the fact that the p67sporozoite stage-specific surface antigen is highly conserved, in contrast to the polymorphic nature of PIM, has been exploited in the construction of a p67-based molecular vaccine. The exact nature of protection is still unknown, but high titres of neutralising antibodies induced by vaccination with recombinant p67 are thought to play an important role. Such high titres are not found during natural infections. The sporozoite surface coat is only exposed for a short time to the circulating antibodies, as association and invasion of a host cell occurs rapidly. If neutralising antibodies per se play a major role in protection titres of neutralising antibodies need to be maintained over long periods to prevent infection. The effect of natural boosting of the neutralising antibodies and the percentage of severe reactions resulting from inadequate protection will be difficult to predict under different epidemiological conditions. It is unlikely that interference solely with the invasion process would be sufficient to provide adaquate protection.

Delayed type hypersensitivity (DTH) responses have been investigated *in vivo* in immune animals with antigens based on schizont extracts from tissue culture. These could be the basis for a practical immune status test but did not give consistent readings (T. Dolan and M. Flowers, personal communication). A recent report on the use of theilerin in *T. annulata* confirmed this (Hashemi-Fesharki *et al.*, 1998).

1.1.5.2. Cell mediated responses

One of the principal effectors of the protective cellular immunity are CTLs directed against the schizont-infected cells. The immune cells responsible for protection through lysis of infected cells belong to the $CD8^+$ T-cell subpopulation and are MHC class-I (MHC-I) restricted and parasite strain specific.

The evidence is based on the following:

Passive transfer with serum from immune cattle was unsuccessful. (Muhammed *et al.*, 1975)

Immunisation with inactivated preparations of autologous infected cells was unsuccessful, but immunity could be achieved by the transfer of lymphocytes between protected and naive chimaeric twins (Emery, 1981a).

Parasite-specific CTLs were found, suggesting their importance in the immune response to ECF (Emery and Morrison, 1980).

Non-specific and specific CTLs are found when immune responses are mounted in an infected animal (Emery and Morrison, 1981c).

Specific CTLs are found transiently in blood of challenged animals at the time of schizont clearance. (Engui and Emery, 1981; Morrison *et al.,* 1987)

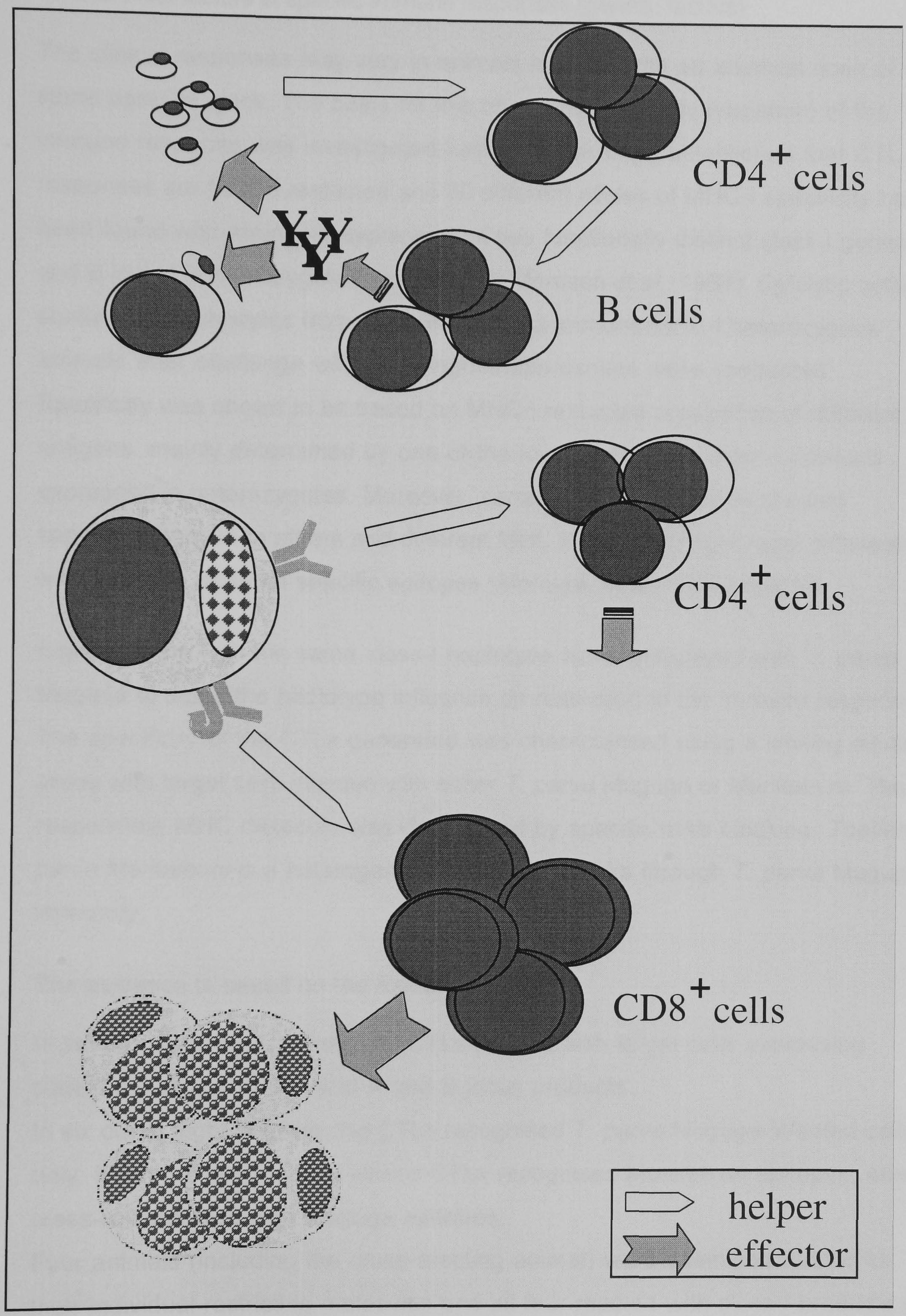
Transfer of responding $CD8^+$ CTLs from a monozygous twin calf provided immunity to the naive twin calf and confirmed the role of $CD8^+$ CTL in *T. parva* immunity (McKeever *et al.,* 1994).

Further study of the mechanisms of the CMI responses to *T. parva* revealed the importance and the nature of $CD4^+$ T cell help in the generation of CTL responses (Taracha *et al.,* 1997). It was shown that this interaction was dependant on close cell-cell contact (Taracha *et al.,* 1997). Brown *et al.* (1990) have shown that *T. parva* infected cells can present endogeneously processed antigen in association with self-MHC and the fact that infected lymphoblasts express high levels of class I and class II MHC molecules (Demartini and Baldwin, 1991) provides circumstantial evidence of good antigen presenting capacities. Parasite-specific $CD4^+$ T cells are generated following immunisation (Baldwin *et al.,* 1987; Brown *et al.,* 1989) and it could be envisaged that *T. parva*

infected cells play a central role by providing priming to both $CD4^+$ and $CD8^+$ T cells. The frequency of CTL precursors (pCTL) in peripheral blood lymphocytes (PBL) of immunised animals is low (1:2,000 to 1:12,000) but increases to 1:1,000 and during 2- 3 weeks after immunisation (Taracha *et al.,* 1995), with levels reaching 1:30 in efferent lymph (McKeever *et al.,* 1994).

Although a parasite-specific $CD4^+$ T cell clone with cytotoxic activity has been isolated in one study (Baldwin *et al.,* 1992), the low prevalence of such clones suggests that these do not play an important role in the immune response.

Fig. 3. Major immune responses in *Theileria parva*.



1.1.5.3. Host factors in specific immune responses (genetic factors)

The clinical responses may vary in animals infected with an identical dose of the same parasite stock. The basis for this observed specificity (variation) of the immune response was investigated further. It has been established that CTL responses are MHC-I restricted and 50 different alleles of MHC-I specificity have been found with abundant expression of two functionally distinct class-I genes (A and B locus) in homozygotes (reviewed by Morrison *et al.,* 1986). Cytolytic activity studies of lymphocytes from *T. parva* Muguga immune MHC-I heterozygous animals after challenge with homologous sporozoites were conducted. Specificity was shown to be based on MHC-I restricted recognition of different antigens, mainly determined by one of the four polymorphic class-I products expressed in heterozygotes. Moreover, certain MHC-I molecules showed superior affinity than others and different MHC molecules might react differently with common or strain specific epitopes (Morrison *et al.,* 1987a and b).

Eight animals with the same class-I haplotype were immunised with *T. parva* Muguga to study the haplotype influence on restriction in the immune response. The specificity of the CTLs generated was characterised using a limiting dilution assay with target cells infected with either *T. parva* Muguga or Marikebuni. The responsible MHC molecule was determined by specific mAb blocking. *Theileria parva* Marikebuni is a heterogenous stock that breaks through *T. parva* Muguga immunity.

The evidence is based on the following:

In seven out of eight animals, all CTLs reacted with target cells expressing class-I molecules of identical A and B locus products.

In six out of eight animals, the CTLs recognised *T. parva* Muguga-infected cells only. From the two animals whose CTLs recognised Marikebuni epitopes, one cross-reacted also with Muguga epitopes;

Four animals (including the cross-reacting animal) were examined further for their individual restricting molecules and all four reacted with class-I products of the B locus only, although one had CTLs to Marikebuni (Morrison *et al.,* 1995b).

These results indicate the existence of a hierarchy of dominance in the recognition of epitopes presented by the different MHC-I molecules (haplotypes). This is seen in the MHC-I molecule expressed by the B locus with its strong tendency to select strain specific antigens in Muguga immune animals. This affiliation is not absolute as this MHC specific molecule could also react with a conserved antigen, shared between the two stocks. Animals with strain restricted CTLs also responded to *T. parva* Marikebuni epitopes after challenge with this stock. It seems likely that some form of antigenic competition among strain specific epitopes drives the CTL-based immune response. There is also evidence that the polymorphism in the T-cell receptor (TCR) repertoires of the host might play a role in the affinity differences towards different epitopes. Some animals might lack good affinity receptors for certain epitopes of *T. parva* strains (Morrison *et al.,* 1995).

In another experiment, the haplotype restriction was studied using different *T. parva* stocks in identical twins. One pair of twins, immunised with *T. parva* Muguga, had a response that was restricted by only one haplotype. Another pair of twins, immunised with a *T. parva* Marikebuni clone, had either a specific response, restricted by another haplotype, or a cross-reactive response, restricted by the haplotype in common with the *T. parva* Muguga immunised twins. From these and related studies, it appears that one haplotype recognised epitopes in most animals, indicating that the response of animals is focused on a limited number of antigen epitopes. This low number was also suggested to explain the remarkable immunodominance often shown by virus-specific CTLs in mice and humans (reviewed by McMichael and Phillips, 1997). The existence of an association between an HLA haplotype and *P. falciparum* protective epitopes has also been reported by Hill *et al.,* (1991). Plebanski *et al.* (1997a) suggested, in the case of *P. falciparum*, that hosts might have a personal epitope preference repertoire of memory T cells, shaped during the first parasite encounter and remaining stable over time. This would mean that individuals might never mount a CTL response against specific epitopes recognised by others (Plebanski *et al.,* 1997a).

1.1.5.4. Parasite-related factors: strain specific immunity

It has been known for many years that animals can be immunised against *T. parva* using infective sporozoites and that immunogenically different strains exist that do not cross-protect (Radley *et al.,* 1975a; Paling and Geysen, 1981; Irvin *et al.,* 1983). Immune responses have been studied *in vitro* and *in vivo* from animals immunised with different immunological strains or clonal populations using a variety of experimental designs. Strain specific bovine helper (Baldwin *et al.,* 1987) and cytotoxic T cell clones (Goddeeris *et al.,* 1986) have been generated as well as cytotoxic T cells recognising common epitopes (Morrison *et al.,* 1987a).

The strain specificity of responses has been related to the different MHC-I haplotypes, as CTL clones restricted by different haplotypes had different strain specific responses (Goddeeris *et al.,* 1990). Limiting dilution assays, using specific CTL clones, were performed in order to study the strain specificity of T-cell clones generated in animals immunised with *T. parva* Muguga and their ability to protect against challenge with a *T. parva* Marikebuni clone. This study showed that six out of seventeen animals (35%) generated cross reactive CTLs and eleven out of seventeen animals had only *T. parva* Muguga specific CTLs and reacted to challenge with Marikebuni. Furthermore, six *T. parva* Marikebuni immunised animals were challenged with *T. parva* Muguga and the study showed that three out of five animals generated cross reactive CTLs and two out of five animals only had specific CTLs and were susceptible to challenge. These results demonstrate the ability of animals to mount both a strain specific and a cross-reacting CTL response. T cells in different animals recognise different antigen epitopes and this highlights the important influence of the parasite antigens in this response (Taracha *et al.,* 1995a).

It can be concluded that immunisation induces strain specific CTL responses in some animals and cross-reactive CTLs in others which correlate with protection. These differences are defined mainly by the genetic make-up of the parasite. There is evidence that the protective response might be determined by a limited number of protective epitopes present on the parasite (Taracha *et al.,* 1995a and

b) as has also been reported in the case of *Babesia bovis* (reviewed by Brown *et al.*, 1994). Epitope selection by different MHC-I molecules could influence specificity of CTLs, but it seems more likely that the immunizing parasite has the most profound influence on CTL specificity (Taracha *et al.*, 1995a and b).

1.1.5.5. Mechanisms of cross-immunity responses

Immune responses to *Plasmodium* are more complex than those to *Theileria* due to major differences in their lifecycles. But extensive data are available from *Plasmodium* which could help to describe some of the common mechanisms that are operating in both infections. Data on CTL responses in viral infections suggest the importance of only a few epitopes in the immune response (reviewed by McMichael and Phillips, 1997). This immunodominance is not explained by higher binding through abundance (Gotch *et al.*, 1987), better affinity (McAdam *et al.*, 1995) or high expression on the cell surface (Tsomides *et al.*, 1994). Yet chance does not play a role, as most individuals with identical human leucocyte antigen (HLA) molecules respond to the same epitopes (Elliott *et al.*, 1993; McMichael and Walker, 1994). Research on CTL responses in viral and other protozoan infections has indicated that several CTL inducing epitopes might be present on the same protein in overlapping form, acting in co-dominance or in an exclusive way (Plebanski *et al.*, 1997a). From research on virus-specific T cell responses, it has become apparent that the generation of a good CTL response is dependent on co-stimulation of CTL cells by Th1 cells. The presentation of CTL epitopes and Th1 stimulating epitopes, not necessarily present on the same protein (Kaneko *et al.*, 1997) will be required and both epitopes need to be presented by the same cell. Moreover both stimulating and inhibiting epitopes might be present and the ensuing linear organisation would determine their mutual interaction (Sercaz and Krzych, 1991).

Research on T cell epitopes in *Plasmodium* is based on synthetic peptides as inducers of proliferation in PBL present in sera from immune hosts. Protective epitope mapping studies of important antigens are underscoring the complexity of interactions between epitopes and different arms of the cellular immune system. Nevertheless, these studies indicated that regions of general antigenic

importance could be identified (Udhayakumar *et al.,* 1995; Kulane *et al.,* 1997). There is no reason to doubt that strain specific epitopes can be presented by the same MHC-I haplotype but could be recognised by different CTLs.

1. The current hypothesis regarding the nature of cross-immune responses in *T. parva* infections is based on the mosaic model (Morrison, 1996). Each stock is composed of a mixture of immunogenic epitopes which might be partly or fully shared or restricted between different *T. parva* stocks. The immune response might be determined by an hierarchy of dominant MHC-peptide combinations giving different responses in animals. This is a consequence of the limited ability of the immune system to mount effective protective reactions to a few highly dominant parasite determinants (Taracha *et al.,* 1995a and b). But the underlying mechanism is not yet understood as it depends on complex interactions between the host haplotype, the TCR repertoire and the parasite strain. Epitopes might be dominant for one haplotype and less dominant for another. Epitope expression might differ between and among different parasite strains. CTL responses can be mono- or oligo-clonal and stable over time, as in HIV, limiting recognition of mutants (Kalams *et al.,* 1994), or polyclonal and preventing immune evasion as in Epstein-Barr virus (Silins *et al.,* 1996). In cases of low cross protection, immunisation could have stimulated a partially protective response, probably involving a low frequency of cross-reactive precursor CTLs (pCTL).

The mosaic concept is based on the observations that field isolates are often composed of genetically heterogenous populations and therefore constitute a mixture of epitopes. This was found in *P. falciparum* (reviewed by Babiker and Wallliker, 1997), in *T. annulata* (Ben Miled *et al.,* 1994), as well as in *T. parva* (*T. parva* Mariakani and Marikebuni) in cattle and in *T. lawrencei* infections in buffalo (Conrad *et al.,* 1987a and b; Toye *et al.,* 1991). In the buffalo, the infection is composed of a mixture of immunogenically different parasite populations, whose components will fluctuate over time (Conrad *et al.,* 1987a and 1989a). Such situations resemble quasi-species definition, as defined in RNA-virus infections (McClure, 1996). The high multiplication rate of a virus, combined with

the sloppy RNA-based copy mechanism causes the generation of a highly diverse virus population, limiting the formation of a population structure with discrete parasite strains. In *T. parva* infections, a similar situation is likely to prevail under epidemiological conditions in which frequent inoculation and uptake of parasite populations composed of multiple genotypes occurs.

It can be envisaged that different genotypes in an inoculum could proliferate sufficiently, especially during primary infections, before effective host immune responses intervene. If there are sufficient chances of being picked up by ticks before being eliminated, multiple genotypes could become prevalent in field populations. In this situation, immunisation with highly heterogenous stocks (the cocktail vaccine approach) would be advantageous as it would involve contact with a larger array of different immunodominant epitopes and hence contribute to a broader immune response. This approach may even alter the hierarchy of immunodominance through bias towards more broadly conserved epitopes. In parasite population mixtures, these might outnumber the specific epitopes and hence influence protective responses towards a broader array of strains.

2. An alternative hypothesis for the nature of cross-protection is the antigenic competition based on the fact that CTLs of different specificities, focused on a limited number of epitopes, are generated in each animal, so that higher levels of specific pCTLs will be present when using homogenous rather than heterogenous strains as a vaccine. A complicating factor in assessing these cross-immunity responses is the possibility that antigen competition might influence restrictive, strain specific responses, giving only a more conserved epitope response on challenge (Morrison *et al.,* 1995a).

This last hypothesis seems to be supported by data from cross-immunity trials in which stocks derived from relatively homogenous field situations, as later defined by molecular characterisation by Bishop *et al.* (1994), gave broader protection than would be expected from their lack of genotype complexity. *Theileria parva* Boleni from Zimbabwe (Irvin *et al.,* 1989), the Katete stock from Eastern Province (Andico project reports) and the Chitongo stock from Southern

Zambia gave a wide cross protection in challenge experiments (Asveza project, unpublished results). These results seem to indicate that the broadly protective epitopes are well conserved under these situations.

The complexity of parasite stocks found in endemic areas might be the result of a strong strain restricted evolution in the CTL response induced by the parasite. This response would be beneficial to the parasite's survival and transmission under conditions where only a few susceptible host species are found in the wild (evolution towards a dominant strain would restrict parasites with similar epitopes for establishment and maintenance of infection in the same host). Cohabitation of parasites in the same host species would only be possible if the components did not show cross-protection, meaning that they should express differences in the epitopes that are important in inducing protective responses. The major mechanisms controlling co-infection must be operative against the pathogenic life cycle stage, the intralymphocytic shizont stage in *T. parva* and the erythrocytic bloodstage in *Plasmodium*. Diverse immune evasion strategies are used by bloodstream stages. They include the generation of multiple immunogenic antigens (*Plasmodium*), gene polymorphism by antigenic variation (PfEMP1 in *Plasmodium*, Roberts *et al.,* 1992) or sequence polymorphism by mutation, conversion or recombination (immunodominant merozoite/piroplasm surface antigen (Tams) in *T. annulata*, Shiels *et al.,* 1995), or a mixture of these strategies (reviewed by Lanzer *et al.,* 1995). Most studies on immune evasion from cell-mediated responses relate to viral infections. In the case of *T. parva*, an hypothesis has been put forward by Dolan (1986a) suggesting the survival of schizont infected cells in sites of low immunological surveillance. Evasion of cell mediated immunity reported in virus diseases often involves pathogen interference with host cell gene expression, altering receptors or communication links between cells. Other mechanisms have evolved based on interactions with intercellular or intracellular communication channels mimicking receptors or cytokines (reviewed by Kotwal, 1996; Marrack and Kappler, 1994). It could be postulated that *T. parva* parasites might downregulate expression pathways as described for other parasites by Hommel

(1997) or communication lines between cells in order to become less conspicuous to the immune system.

In the most recent hypothesis based on studies with bacterial infections, the immune responses were identified as the major selection pressure (driving force) in determining the subdivision of the field population structure into discrete strains (Gupta *et al.*, 1996). Depending on the transmission intensity, reflecting the epidemiological situation, the complexity of strain composition might vary. This composition might involve clones characterised by different immunodominant epitopes and lacking in cross-immunity properties. Determination of the antigens characterising field strains could be a useful approach to revealing important protective proteins.

It can be concluded that protection is mainly a function of the complexity of parasite stocks rather than host determined factors, but the role of the main factors contributing to this complexity, such as recombination and the reservoir function of carriers in cattle or buffalo, have not been defined. It is clear that cellular responses are important but the antigens involved have yet to be identified.

1.1.5.6. Protective antigens

The search for protective antigens giving rise to cell mediated immunity has proved difficult because of their complex nature. Epitopes recognised by CTLs are known to be short peptides of 10 amino-acids, generated inside the host cell from secreted parasite proteins and expressed in association with MHC-I molecules on the membrane of the infected cell. These short peptides are not recognised by mAbs and are detected only by specific CTLs.

The availabilitiy of techniques for elution and characterisation of antigenic peptides directly from the MHC-I groove of infected cells (Rammensee *et al.*, 1993) looked very promising. But it has not given the expected success in the identification of the target antigens for *T. parva*. More indirect techniques, based on transfection with cDNA or artificial induction of protective CTLs have been

used for *T. parva* but have also turned out to be cumbersome and unsuccessful to date (V. Nene, personal communication). Determining putative CTL epitopes in antigenic sequences by computer algorithm is another approach. This is built on an ever growing database of MHC-I specificities, anchor amino-acid interactions and the protective potential of molecular vaccines (Margalit, 1987). A recent technique using enzymatically labeled MHC-tetramers complexed with relevant epitope peptides can visualise and quantify T-cell clones displaying the appropriate TCR (Altman *et al.,* 1996). This approach allows the study of specific T-cell responses and the epitopes responsible for the expansion of these T-cell clones (McMichael and O'Callaghan, 1998).

Although a polymorphic immunodominant (PIM) antigen has been found on the surface of sporozoites and macroschizonts, and characterised by mAbs (Lalor, 1983; Minami *et al.,* 1983), it is not known if this molecule plays a role in cell mediated responses. Further studies using *T. lawrencei* infected cells identified one mAb that was associated with the infections. This turned out to be a host cell response and not a parasite product (Newson *et al.,* 1986). Antibody based searches for antigen on the surface of infected cells, including complement and antibody dependent cell mediated lysis, were all negative (Creemers, 1982). Molecular epidemiology could contribute towards this search by studying field population genetic (mating) structures. From these data, immunodominant protective antigens could be identified that might be responsible for the underlying strain structure and a comparison of their different alleles could be undertaken (Gupta *et al.,* 1996).

Clear protective responses have been found directed against the p67 sporozoite stage-specific surface antigen. The sequence of p67 is highly conserved and makes this a promising candidate for a vaccine antigen. Its conserved nature is an indication that this protein is not under immune selection (Nene *et al.,* 1996), compared to its more diverse equivalents in *T. annulata* (Katzer *et al.,* 1994) or in *P. falciparum* (Lockyer *et al.,* 1989; Udhayakumar *et al.,* 1994 and 1997). An explanation for this sequence conservation might be sought in a weak immune response to this surface antigen due to the short extracellular stay of the

sporozoite in the host (Fawcett *et al.,* 1982), together with the rapid disappearance of the sporozoite surface coat from the host cell membrane once in the animal. A molecular engineered vaccine has been developed (Musoke *et al.,* 1992) and laboratory trials using sporozoite stabilate challenge resulted in 70% of the immunised cattle being protected. Field trial results are being awaited. In *Plasmodium*, the role of $CD8^+$ T cells and evidence of CTL epitopes on the circumsporozoite protein (CSP) (Good *et al.,* 1988) are additional indications of a different role of the p67 equivalent in malaria.

1.1.5.7. Cytokine mediated responses

Cytokines are host cell products generated in response to infection and in a number of protozoan infections, parasitized cells may also produce interferons (Preston *et al.,* 1993). It is known that the cytokine network plays an important role in controlling the replication of parasites. *Theileria parva* -infected cells have been shown to secrete IFN-γ (DeMartini and Baldwin, 1991) and IL-2 (Heussler *et al.,* 1992). Generally, their effects can be categorized into three functional groups: (1) parasite-inductive cytokines; (2) parasite-suppressive cytokines; (3) cytokines with both activating and inhibiting capacities (Vicenzi *et al.,* 1997). There is growing recognition that the immune responses and pathology in *T. parva* are driven by parasite induced cytokine responses.

The cytokine environment has also been considered as the major variable influencing Th1/Th2 skewing of the immune response (Constant and Bottomly, 1997). Glycoconjugates and glycosyl phosphatidylinositol (GPI) anchors, extracted from protozoa are important molecules in the induction of cytokine synthesis. The mechanisms of these cytokine mediated responses have been studied more extensively in *T. annulata* than in *T. parva* infections. T cells in the draining lymph node are activated 'non-specifically' by parasite infected cells. T-cell dynamics are different from those seen in mitogen stimulation, but an antigenic determinant (superantigen) as in *Trypanosoma cruzi*, reacting with the majority of Vß receptors of T cells has so far not been identified. Reverse transcriptase PCR (RT-PCR) studies have demonstrated a direct correlation between mRNA levels of IL-1 α and the degree of non-specific T cell proliferation

(Brown *et al.*, 1995). Both $CD4^+$ and $CD8^+$ T cells are induced in this non-specific proliferation, leading to failure of the immune response to control the infection as implied by the loss of germinal center morphology in infected lymph nodes (Campbell, 1995).

Protective immune responses against intracellular protozoa are usually mediated by Th1 responses, giving rise to specific CTLs and IFN-γ mediated induction of NO by macrophages. These responses have been reported for both *T. annulata* and *T. parva* infections (Visser *et al.*, 1995). The specific effector arm of the immune response seems to be modified by *T. annulata*. The IFN-γ production does not correlate with protection against this parasite as infected cells flourish during its peak production and only very small amounts of IFN-γ are produced at the time of the effective immune response in immunized animals. Expression of MCH-II antigens is increased, allowing lethal disease to progress rapidly. The parasite seems to have developed a survival strategy by not only improving the cellular environment for optimal replication, but also disrupting the normal immune responses by uncontrolled IFN-γ production (Campbell *et al.*, 1997).

In *T. parva* infection the situation appears to be more complex. It has been shown that there is high IFN-γ production both *in vitro* and *in vivo* (Malu, 1992) upregulating lymphoproliferation and T-cell mediated cytotoxicity. Using RT-PCR, cells infected with *T. parva* have been found to produce variable patterns of cytokine responses, including IL-1 alpha, IL-2, IL-4 and IFN-γ, but consistently high IL-10 levels (McKeever *et al.*, 1997). It was suggested that this IL-10 upregulation might well interfere with normal $CD4^+$ and CTL immune responses, resulting in an immune deficiency related pathology. Increased IL-10 production stimulates the proliferation of non-cytolytic $CD4^-$ $CD8^-$ cells and their products yielding the typical pathological signs associated with a Th2 type response. It has been claimed that the aberrant production of IL-1 alpha by cells infected with the parasite is a major signal for the induction of non-specific T cell proliferation (Brown *et al.*, 1995). This might inhibit generation of effector T cells (reviewed by Morrison *et al.*, 1995b). Survival in a *T. parva* infection can be

viewed as a race between the parasite and the host immune system. The parasite seems to manipulate its environment for fast multiplication while depressing effective immune responses. This is underscored by the extensive activation of T cells to blast formation reported in *T. parva* infections, where only 1 in 30 blasting cells is found to be infected (Emery, 1981b).

An IL-2 autocrine pathway was discovered in infected cell lines (Dobbelaere *et al.*, 1990), stimulating cell activation by producing variable concentrations of IL-2. By blocking this pathway, there was a marked reduction in growth rate but it was not sufficient to stop proliferation. The role of increased IL-2R α and IL-2 upregulation in infected cells, indicative of an autocrine loop, has not been confirmed in the majority of infected cell lines studied by McKeever *et al.* (1997). These results suggest an important role for other stimuli in the process. Carrington *et al.* (1995) proposed a multi-pathway hypothesis to explain the interaction between host cell and *T. parva* parasite, as found in *in vitro* systems.

There are indications of involvement of other possible parasite dependent stimuli (Palmer *et al.*, 1997). Elevated levels of casein kinase II (CKII) are found in *T. parva* transformed cells (Ole MoiYoi *et al.*, 1993). CKII is an ubiquitous enzyme, suspected to play a pivotal role in cell differentiation, proliferation and apoptotic processes (reviewed by Cano and Mahadevan, 1995). The protein kinase family comprises of mitogen-activated enzymes leading to selective gene expression and DNA multiplication, under tight control of regulating molecules. It has been found that mutations or loss of phosphorylation sites in the CKII substrates induces oncogenic transformation. CKII is composed of four subunits, one of which was found in *T. parva* parasites. Dysregulation of this subunit (CKII α) resulted in development of neoplasms. The exact mechanism of proliferation is not known, but there are indications implicating either direct interference in the CKII pathway through uncontrolled CKII alpha secretion (Ole MoiYoi, 1995) or upstream modulation of CKII production (Grab *et al.*, 1998). It is known that G-proteins are involved in trans-membrane signaling events, modulating the production of CKII and some mutant forms of G-proteins have been implicated in oncogenesis. On the other hand, antigen-dependent proliferation of T cells is driven by CKII activity triggered through IFN-γ stimulation

and cell-cell contact, but several kinases need to be activated for upregulation of IL-2 and IL-2R expression (Galley *et al.*, 1997). Results so far indicate that *T. parva* dependent proliferation uses pathways that can be stimulated continuously in contrast to the antigen-receptor mediated one. The complexity of the various pathways identified so far have led to the formulation of a competition model in which different parasite-infected cells compete with rapidly cycling cells.

Similar parasite-induced mechanisms of immune evasion have been described in *Toxoplasma gondii* infections in which the skewing of the immune response towards a Th2 response by parasite induced IL-10 production inhibits the normal production of IFN-γ by natural killer cells when stimulated by parasite antigens (Gazinelli *et al.*, 1991).

1.1.6. Epidemiology

The epidemiology of vector-borne diseases is determined mainly by the vector ecology, the host type and density, and the life cycle of the parasite.

1.1.6.1. Vector ecology

Climatic conditions have an important influence on tick population dynamics and hence the transmission possibilities, creating different epidemiological situations over the entire *T. parva* distribution range (Short and Norval, 1981). Three different phenologies of the tick vector, *R. appendiculatus*, are recorded in relation to rainfall and temperature. They might overlap in the transition zones where, due to significant climatic variation, an alternation between the different adjacent tick generation patterns might occur. The phenology is defined by whether there is one, two or many tick generation(s), determining the occurrence of adult (AA) and nymphal (NN) tick transmission, and the probabilities for larval (LL) and NN pick-up either from acute or chronic infections in the bovine host. This will give rather complex transmission patterns in most ECF affected regions. Diapausing behaviour in ticks from southern Africa has been described by several authors (Short *et al.*, (1989) for Zimbabwe; Berkvens, (1990) for

Eastern Zambia; Pegram and Banda, (1990) for Southern Zambia) and adds to the complexity of transmission (Billiouw, 1997). The eastern African situation is characterised by year round transmission due to the presence of AA ticks throughout the year. The transition zone, where climatic conditions are not favourable for a year round presence of AA, a rainy season and a dry season transmission peak might prevail, while diapausing behaviour in adults influences the transmission dynamics and contributes towards higher numbers of acute infections. The occurrence of only one tick generation a year in the southern part of the *R. appendiculatus* range reduces the transmission period further to four months, coinciding with the rainy season (Norval *et al.,* 1991). *Rhipicephalus zambesiensis* ticks replace *R. appendiculatus* where conditions become dryer, but their role in the epidemiology of *T. parva* in dryer climate zones has not yet been defined.

1.1.6.2. Host type and density

The host population dynamics are probably very similar over the entire ECF range and are determined mainly by the degree of intensification of livestock management. This can be split into traditional cattle management and various forms of farm-based or more intensive management. The African cattle population consist of different breeds, which show differences in tick resistance (Rechav and Zederberg, 1986; Norval *et al.,* 1988) and parasite susceptibility (Radley, 1978; Dolan and McHardy, 1978). The main breeds or types are Zebu (*Bos indicus*), European (*Bos taurus*) and Sanga (Zebu x *Bos taurus*, Hamitic Longhorn), with various degrees of cross-breeding among these types. *Bos taurus* breeds are the most susceptible to tick infestation and *T. parva* infection, whereas Sanga and Zebu type cattle from endemic areas show an high degree of innate resistance against *T. parva* infections (Paling and Geysen, 1981; Moll *et al.,* 1986). Breed differences in resistance to *R. appendiculatus* might contribute substantially to the efficiency of *T. parva* transmission (Walker *et al.,* 1990). The present investigations are related to the traditional livestock keeping sector in Zambia, using a mixture of Zebu, various crosses of *Bos taurus* and *Bos indicus* cattle (Eastern and Southern Province) and Sanga type cattle (Southern Province).

1.1.6.3. Parasite life cycle

An important factor in the epidemiology is the persistence of the parasite in its various hosts. Premunition by means of a carrier state is a consistent feature of most vector-borne protozoan diseases. There is no transovarial transmission of *T. parva* through ticks, and although a sterile immunity was the established dogma for a very long time (Theiler, 1904; Wereyon, 1926), a carrier state in the bovine host was suspected from field observations (Koch, 1904; Bevan, 1924; Viljoen, 1930). The carrier state was confirmed by tick transmission studies (Dolan, 1986a; Young *et al.,* 1986) and is generally accepted now, although it does not occur in the much studied *T. parva* Muguga stock as has been shown by tick transmission (Young *et al.,* 1990) or when PCR amplification was attempted (Bishop *et al.,* 1992). The absence of a carrier state is unusual but not unique. Occasionally more complex stocks, such as *T. parva* Pugu, have been found that do not induce a carrier state (Dolan, 1986a and b). The mechanism(s) involved in the maintenance of the carrier state are not known, although more evidence points in favor of the survival of the schizont stage in sites of low immunological surveillance (Dolan *et al.,* 1986a). Its importance was re-emphasized when *T. parva* parasites were isolated, using standard tissue culture techniques, from animals three and seven months after vaccination by the infection and treatment method (ITM) (Kariuki *et al.,* 1995). Although *T. parva* schizogony in red blood cells has been reported (Conrad, 1986; Fawcett, 1987), it seems unlikely that piroplasms maintain the carrier state. The low rate of replication is more compatible with gametogenesis (see 1.1.4.).

Parasite infectivity and survival in the tick is temperature dependant and limited at both cold and warm extremes (Young and Leitch, 1981). Survival ranges in adult ticks (AA) are between nine months and two years (Shaw and Young, 1994) with shorter periods for nymphae (NN) (Newson *et al.,* 1984). Quantification of infection rates in groups of ticks is usually obtained by estimating the abundance, the mean number of infected acini per tick. The abundance in nymphal ticks fed as larva on an animal with an acute infection is three to twenty times less than observed in AA ticks. Following pick up from a carrier infection, nymphal abundance might differ by a factor 400 in comparison

with abundance in adults. It was not possible to infect larvae (LL) with some *T. parva* stocks, although NN ticks applied simultaneously gave rise to good infections in AA (Ochanda *et al.,* 1996). There are also indications that differences between tick populations (Ochanda, 1994) and interactions between parasite stocks and tick populations are factors influencing abundance rates in ticks (Shaw and Young, 1994; Ochanda *et al.,* 1998). There results indicate that LL ticks might or might not become infected and when infected, show a low abundancy. Nymphal ticks show higher abundance rates than larval ticks, but lower than adult ticks. Factors influencing tick infection rates have been analysed from data from large numbers of cattle used for stabilate production (Young *et al.,* 1996). A heritable basis of tick susceptibility to *T. parva* infection has been shown and selection for more or less susceptible lines is possible (Young *et al.,* 1995).

Differences in abundance are also observed between the sexes in *R. appendiculatus*. Female ticks develop higher infection rates and levels, although nymphs that moult into male adults ingest more blood than moulting females. The major difference between the sexes relates to a higher number of "e" cells in type III acini in the salivary glands of female ticks (Young *et al.,* 1981) that have been shown to be infected preferentially by *T. parva* sporozoites (Fawcett *et al.,* 1982). The 'e' cells are situated at the periphery of the gland and so may be more available to become infected by kinetes on their journey through the haemolymph to the salivary gland (Shaw and Young, 1994). Walker (1990) speculated that invasion might be selective through the recognition of specific receptors on the cells of the salivary gland.

Transmission in the various epidemiological situations is best quantified by the entomological inoculation rate (EIR). This parameter gives the number of infective bites per animal per year for a certain region and can be approximated from tick infestation on cattle and *T. parva* infection rate data. This allows a comparison of transmission intensities between regions and between different diseases, and is fundamental to the interpretation of parasite derived population genetic data.

The EIR can be calculated from AA infestation rates, if some basic assumptions are made.

a=AA counts/year x 2

n=NN counts x 5

i_a=%infected AA ticks

i_n=%infected NN ticks

r = host tick-resistance coefficient

$$\mathbf{EIR = (a\ x\ r\ x\ i_a\ )+ (n\ x\ r\ x\ i_n\ )}$$

Feeding time and therefore the presence on the host differs between the sexes (a male/female sex ratio of two) and the survival percentage between different stages has been estimated as 0.1 (Walker *et al.,* 1981a). A realistic estimation of tick infestation from monthly collection data can be obtained by multiplying the observed AA ticks by two and NN ticks by five (D.L. Berkvens, personal communication). The host resistance coefficient to tick infestation is determined mainly by the cattle breed and the age of the animal. Approximately 50% (range 45-60%) of the total *R. appendiculatus* numbers applied on resistant cattle feed successfully as reported by De Castro *et al.* (1985). The EIR can be calculated, if the prevailing infection rates in ticks for that region are known. The tedious work of tick dissection as the method of determining tick infection rates has limited the number of field ticks analysed as these generally show low abundance, often with only one infected acinus. The results are also compromised if care is not taken to obtain the entire salivary glands. An additional complicating factor is that there is no simple method for discriminating between *T. parva* and *T. taurotragi* infected salivary glands (Young *et al.,* 1980; Voigt *et al.,* 1995). *Theileria taurotragi* has been reported to be present also in the Southern Province of Zambia (Jongejan *et al.,* 1986). It is hoped that a better quantification of tick infection in the field can be obtained with new molecular biology techniques (Chen *et al.,* 1991, Bishop *et al.,* 1992).

Tick infection rates in field ticks have been reported by several authors and indicate low infection rates with low infection intensities (one infected acinus) in most ticks, and a single tick showing high intensity infections. The infections in ticks show a negative binomial distribution, characteristic of vector infections. Young *et al.* (1986) reported a 1.1 % (n=631) infection rate in field ticks from an holo-endemic region in Kenya (shore of Lake Victoria) with six showing a single infected acinus and the remaining tick showing six infected acini. Tick infection rates (N=493) in field ticks collected from animals and vegetation were found to be 1.5% in an endemic area in Kenya (Walker *et al.*, 1981a). Tick infection rates in the Transmara area in Kenya, where a holo-endemic situation prevails, ranged between 1.9-2.6% (n = 1107) with 56% showing only one infected acinus (Moll *et al.*, 1986). Kariuki *et al.* (1995) found a mean infection rate of 1% (range 1-14% in a total of 10 batches of 100 ticks each) in ticks engorged on carrier animals, three and seven months after immunisation with *T. parva* Marikebuni. The infection in these ticks consisted of only a single infected acinus. Subsequently, data obtained from limited field surveys on tick infection rates under varying epidemic situations were all in the same range (Walker *et al.*, 1981a).

The epidemiology of *T. parva* is determined mainly by climatic conditions and host density, and that under more marginal conditions, adaptations such as diapausing behaviour of the tick or infection rate differences in the tick (vectorial capacity) and in the host (resistance) might become important factors. There is an urgent need to complement the crude data on tick infection rates from the field with more accurate data using better parasite detection, discrimination and quantitation techniques.

1.1.7. Virulence

1.1.7.1. Mechanisms

Virulence of pathogens can be the result of different factors and mechanisms (reviewed by Frank, 1996). Virulence is often related to the capacity of parasites to be transmissible (which defines parasite fitness), linked directly to the rate of reproduction and quantified by the parameter Ro (Anderson and May, 1982). In

the case of vector-borne parasites, transmissibility is determined by the vector density, the duration of infectiousness in the host and year round prevalence influencing the possibilities of being picked-up. Under good transmission conditions strains may replicate at higher rates, producing infective stages earlier in the disease process and thereby increasing the number of life cycles over time. This allows for the evolution to higher levels of virulence as long as transmissibility is not affected (Ewald, 1983). The level of virulence will be controlled by natural selection resulting in a virulence optimum related to the different epidemiological situations. The coupling of virulence with transmission implies a high host mortality that will have a negative influence on parasite survival. For survival a parasite must evolve towards an intermediate degree of virulence that will maximise transmissibility.

Variations in pathogenicity may also be due to differences in the immune responses of the host (Antia *et al.,* 1994), often influenced by genetic differences between parasite populations. It has been speculated that *T. parva* infections are the result of a race between the parasite and the immune response of the host (Emery and Morrison, 1980), probably driven by parasite induced production of cytokines (reviewed by McKeever *et al.,* 1997). Hence parasites might differ in their ability to influence the Th1/Th2 balance. Evidence for this is derived from the fact that parasites inducing a milder form of ECF often produce lower parasitaemias but have a significant influence on the haematocrit (J. Brandt, personal communication). It is not clear if this is due to a slower division of the parasite or an altered genotype.

Parasite dose plays a crucial role in the expression of virulence (Jarrett *et al.,* 1969; Dolan *et al.,* 1984) and must be taken into account when evaluating virulence differences. It is also likely that host evolution contributes to the evolution and expression of virulence (reviewed by Ebert and Herre, 1996). The interactions might be complex involving not only transmission intensities, but also dominant and stage specific immune responses, making predictions and comparisons hazardous (Cebula and LeClerc, 1997). Differences in virulence have been reported for various *Theileria* species (*T. mutans*, Irvin *et al.*, 1972 and Moll *et al.*, 1981; *T. annulata*, Darghouth et al. 1996a, *T. parva*, Barnett,

1957). There have been many reports on the isolation of apparently mild *T. parva* stocks from the southern and the eastern parts of its range (the Icely isolate (Kenya), Barnett and Brocklesby, 1961 and 1966; Mandali (Zambia), Musisi, 1986; Lanet (Kenya), Mbogo *et al.,* 1996; Ardlui, Glenfarg (Zimbabwe), Koch *et al.,* 1988a; Boleni (Zimbabwe), Koch *et al.,* 1988b; Hove *et al.,* 1995). However, it is difficult to determine if the reported low virulence of these stocks was due to specific strain related factors or to dose. There are strong indications that dose determined the mild character of the Icely stock, as higher challenge doses increased the virulence of this isolate (Barnett and Brocklesby, 1966). Apart from the lack of good characterisation tools at the time, making it difficult to evaluate these reports, the prevailing concepts in virulence expression are all based on dynamic interactions. These concepts, in which virulence is seen as an expression of an unstable optimum dependent on parasite interactions with their environment, question the justification of the use of mild stocks in region-wide vaccination. It seems that only virulence manipulation under unnatural conditions and provoking 'stable' alterations, probably due to genome changes, might prove essential in this respect as the complex history on *Babesia* vaccine development has shown (Dalrymple, 1992; Bock *et al.,* 1995; Callow *et al.* 1997; Lew *et al.,* 1997).

1.1.7.2. Dynamics of virulence evolution

Important differences exist between an endemic and an epidemic disease situation (Frank, 1996; Claessen and de Roos, 1995). Generally, the evolution of virulence during epidemics is influenced by host densities and transmission efficacy. In endemic situations, the duration of infection is the most important factor and virulence will be less influenced by opportunities for transmission. Translated to the ECF situation, the evolution will be towards an intermediate virulence in an endemic situation as ample transmission possibilities exist but high host mortality and tick mortality due to high numbers of parasites in the salivary glands would counterbalance these (Young *et al.,* 1995; Tempia *et al.,* 1997). Whereas in an epidemic situation, vector density is the main limiting factor and the evolution of virulence will be driven towards milder strains, giving a long duration of infection in the host and reasonable infection rates in ticks. It

has been suggested that situations favoring co-infection (van Baalen and Sabelis, 1995) and superinfection (Nowak and May, 1994) will lead to increased virulence. But the virulence profiles are different in co-infection where parasite populations become monomorphic in relation to virulence level, while in superinfection, various levels of virulence might evolve.

Under this hypothesis, variation in virulence in *Plasmodium* is thought to have evolved due to lack of competition between various strains as weak common immune responses are elicted. In such situations, superinfection with different strains will be common. Strong competition will only exist between strains as a result of shared immune responses. In this case, strains will only coexist if they have a very similar Ro and similar virulence. Maintenance of heterogeneity in parasite virulence would be possible only if there was a virtual lack of effective cross-immunity between strains of different virulence (Gupta et al. 1994). In reality it might be expected that different effects of parasite phenotypes that correlates with virulence will give a non-linear relationship between transmissibility and virulence (Gupta *et al.,* 1994). In the case of *T. parva* it can be predicted that there will be less heterogeneity in virulence due to the high level of cross-immunity between parasite populations in the field.

1.1.8 Control by immunisation

Immunisation using infected cattle tissues (Spreull, 1914), cell culture (Brown, 1981) and infection and treatment (Cunningham *et al.,* 1973) have all been used to provide protection in *T. parva*. The last method has been developed into a practical vaccination method that can control *T. parva* and is based upon the use of cryopreserved sporozoite stabilates. Wilde *et al.* (1968) showed the benefical use of long-term administration of tetracyclines in inducing immunity under natural exposure in the field. An infection and treatment method has been developed based on the use of a titrated suspension of sporozoites, kept as stabilates in liquid nitrogen. A long-acting tetracycline is given at the time of infection to control the resulting *T. parva* infection (Radley *et al.,* 1971). Each stabilate is produced from large batches of ground-up infected *R.*

appendiculatus adults and characterised by defining the immunising dose in a bovine titration experiment.

It has been known for a long time that the infection and treatment method (ITM) does not always provided complete protection in cattle, if they are challenged with a different immunological stock either naturally or experimentally (Radley *et al.*, 1975a; Paling and Geysen, 1981; Irvin *et al.*, 1983). In a series of cross-immunity trials in eastern Africa it was shown that a cocktail or mixture of isolates, using a limited number of strains, could give broad protection against cattle-derived isolates (Radley *et al.*, 1975). However many breakthroughs occurred in areas where cattle had contact with buffalo through shared grazing (Radley *et al.*, 1979). It is generally accepted that these breakthroughs were due to exposure to new *T. parva* antigenic types from the buffalo population. The cocktail developed in East Africa was advocated as an effective control measure for application throughout the whole ECF region. Preliminary cross-immunity trials in Zambia showed that it did not protect against isolates originating from the Eastern Province (Geysen, 1986). Eleven cattle, immunised by the ITM method with the Muguga cocktail were challenged 30 days after immunisation with the homologous stocks to verify their immune status. Three groups of cocktail immunised animals were challenged with three Eastern Province stabilates (*T. parva* Katete (4 animals), Lundazi (4 animals) and Genda (3 animals) together with three, two and two controls respectively. Seven cocktail-immunised animals showed reactions: two had severe reactions with one death, one had a moderate reaction and three animals showed a mild reaction (see next paragraph for criteria of reactions). Five immunised animals showed no reaction. All control animals except one had to be treated with an anti-theilerial drug. Zambian isolates gave good protection in cross-immunity trials using the cocktail as challenging stock (BADC and Asveza South project reports), but differences were found in cross-immunity trials between stocks from the Eastern and Southern Provinces (own results and Asveza South project reports).

A review of the data on cross-immunity trials reported in the literature, data generated in Zambia and own data (Geysen and Brandt, unpublished results),

combined with mAb profiles of the stocks used gives a good idea of the immunogenic and antigenic diversity found in *T. parva*. The suggested hierarchical ordering of parasite stocks based on cross-immunity studies (see below) is intended to provide guidance for further research on antigenic diversity and its relation to protection. In describing the cross-immunity data, the challenge reactions were classified (Irvin *et al.*, 1989) as follows: no reaction (NR) when neither pyrexia nor parasites were detected following challenge, mild reaction (MR) when pyrexia was less than 6 days and the schizont index (MSI) was less than 0.001, moderate reaction (MOR) when pyrexia was between 6 and 12 days and the MSI less than 0.01 and severe reaction (SR) when pyrexia was more than 12 days and the MSI of 1-10%. Animals with no reaction or mild reactions were classified as protected. It has to be stressed that some data are derived from single animal trials, but have been included if these resulted in breakthrough reactions, characterised by moderate or more severe reactions. Cross-immunity trials have to be interpreted with some caution. Many cross-immunity studies presented here were conducted with stocks that have been characterised as a mixture of different *T. parva* genotypes. Often different sporozoite stabilates of a stock were used which might vary in sporozoite density and component composition in relation to the parent stock. But it is felt strongly that even one breakthrough reaction, experienced in cross-immunisation studies following homologous challenge and lethal heterologous challenge doses, provides a good indication of immunological differences between two stocks. The list of cross-protection studies presented below is by no means complete. A number of cross-immunity studies have been conducted with various other stocks from Zimbabwe and East Africa that have not been reported but are included here because the data are sound.

Table 1. *Theileria parva* immunisation and challenge data

Immunising stock	Challenging stocks	No. of animals used	ECF reactions	Breakthroughs	Cross-protection	Refs
T. parva Muguga	*T. parva* Uganda		1	1	No	Uilenberg *et al.*, 1982
	T. parva Uganda	7	1 MR/ 5 SR	5	No	Morzaria *et al.*, 1986
	T. lawrencei	6	6 SR	6	No	Radley *et al.*, 1975
	T. parva Schoonspruit	1	NR	0	no	Uilenberg *et al.*, 1982
	T. parva Nyakizu	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Boleni	1	NR	0	no	Uilenberg *et al.*, 1982
	T. parva Marikebuni	6	2 MR/ 4 SR with 2 D	4	No	Irvin *et al.*, 1983
	T. parva Kilifi	4	NR	0	yes	Irvin *et al.*, 1983
	T. parva Mavueni	6	2 MOR	2	±	Irvin *et al.*, 1983
	T. parva Kiambu1	5	3 SR	3	No	Radley *et al.*, 1975b
Muguga cocktail	*T. parva* Katete	21	6 MR/7 SR with 4 D	7	No	Geysen, 1986 and Lynen, 1991
Muguga cocktail	*T. parva* Chitongo	20	MR	0	yes	Asveza data
T. parva Kiambu5	*T. parva* Uganda	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Nyakizu	1	1	1	No	Uilenberg *et al.*, 1982
	T. lawrencei	4	4	4	No	Radley *et al.*, 1975
	T. parva Kiambu1	5	3 SR	3	No	Radley *et al.*, 1975
T. parva Zanzibar North	*T. parva* Zanzibar South	2	2 MOR	2	yes	Morzaria *et al.*, 1986
T. parva Zanzibar South	*T. parva* Mariakani	4	NR	0	yes	Morzaria *et al.*, 1986
	T. parva Marikebuni	4	3 MR	0	yes	Morzaria *et al.*, 1986
T. parva Pemba	*T. parva* Mariakani	2	2 D	2	No	Morzaria *et al.*, 1986
	T. parva Marikebuni	2	1 MR	0	yes	Morzaria *et al.*, 1986
	T. parva Mnarani	12	2 MR/ 8 SR with 5 D	10	No	Morzaria *et al.*, 1986
T. parva Pemba/ Mnarani	*T. parva* Mariakani	4	4 MR	0	yes	Morzaria *et al.*, 1986
	T. parva Marikebuni	4	2 MR	0	yes	Morzaria *et al.*, 1986
T. parva Boleni**	*T. parva* Muguga	10	2 MR	0	yes	Irvin *et al.*, 1989; Hove *et al.*, 1995
	T. parva Marikebuni	11	MR/1 SR with 1 D	1	No	Irvin *et al.*, 1989
	T. lawrencei Nanyuki	6	2 MR	0	yes	Irvin *et al.*, 1989
	T. lawrencei Ngong1	7	2 MR	0	yes	Irvin *et al.*, 1989

	T. parva Mariakani	7	2 SR with 1D	2	No	Irvin *et al.*, 1989
	T. parva Nyakizu	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Pugu1	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Uganda	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Kasoba	6	6 SR	6	No	Hove *et al.*, 1995
	T. parva Serengeti Tr	7	3 MR/ 2 MOR	2	±	Uilenberg *et al.*, 1982; Hove *et al.*, 1995
T. parva Nyakizu	*T. parva* Uganda	1	NR	0	yes	Uilenberg *et al.*, 1982
T. parva Uganda	*T. parva* Nyakizu	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Kiambu5	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Serengeti Tr	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Muguga	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Boleni	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Pugu1	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. lawrencei (Manyara)	1	NR	0	yes	Uilenberg *et al.*, 1982
T. lawrencei (Manyara)	*T. parva* Nyakizu	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Uganda	1	1	1	No	Uilenberg *et al.*, 1982
T. parva Pugu1	*T. parva* Nyakizu	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Boleni	1	NR	0	yes	Uilenberg *et al.*, 1982
	T. parva Uganda	1	1	1	No	Uilenberg *et al.*, 1982
T. parva Schoonspruit	*T. parva* Nyakizu	1	1	1	No	Uilenberg *et al.*, 1982
	T. parva Uganda	1	1	1	No	Uilenberg *et al.*, 1982
T. parva Tanzania stocks*	*T. parva* Kasoba	5	NR	0	yes	Musisi *et al.*, 1994
T. parva Marikebuni	*T. parva* Kilifi	5	NR	0	yes	Irvin *et al.*, 1983
	T. parva Mariakani	4	NR	0	yes	Irvin *et al.*, 1983
	T. parva Mavueni	2	NR	0	yes	Irvin *et al.*, 1983
T. parva Serengeti Tr	*T. lawrencei*	4	2 SR	2	No	Radley *et al.*, 1975
T. parva Katete	*T. parva* Chitongo	5	3 MR	0	yes	Geysen and Brandt
	Cocktail	10	3 MR/1D Haertw	0	yes	Lynen (pers.comm)
T. parva Chitongo	*T. parva* Katete	7	1 MR	0	yes	Geysen and Brandt
	Cocktail	20	MR	0	yes	Asveza data

**T. parva* SAO Hill and W.Kilimanjaro. ** Boleni stocks used by Uilenberg *et al.* (1992) were different from those used by Irvin *et al.* (1989)

It can be concluded from these data that the *T. lawrencei* stocks are generally antigenically different from the *T. parva* stocks. The diversity in *T. parva* field stocks seems limited. *Theileria parva* Marikebuni, Uganda, Nyakizu and Kasoba were stocks which differed significantly in their cross-immunity profile from the others. There is some indication that cross-immunity might act in both directions, which might suggest the presence of dominant identical or cross-reactive epitopes in the protective antigen(s) of these stocks. Evidence for this was presented by Kariuki *et al.* (1990) who compared the cytotoxicity of CTLs generated in genetic related animals infected with different *T. lawrencei* and *T. parva* stocks. Target cells were produced from two cell-lines infected *in vitro* with different *T. lawrencei* and *T. parva* sporozoites. *Theileria parva* Marikebuni-induced CTLs responded best to epitopes expressed by all stocks and most *T. lawrencei* CTLs recognised epitopes of the five different *T. lawrencei* stocks. *Theileria parva* Muguga was the least reactive and the cytotoxicity induced by *T. parva* Mariakani, Boleni and Uganda killed half of the target cells. The interpretation of these results was difficult as evidence for the presence of mixed parasite populations was demonstrated in some of the stocks generating CTLs with distinct epitope specificities. Similarity between stocks was reported by Sugimoto *et al.* (1989) who characterised schizont-derived protein profiles using two-dimentional gel electrophoresis. This enabled differentiation of over 200 different proteins that yielded identical profiles for *T. parva* Uganda and Mariakani, and similar profiles for *T. parva* Boleni. In contrast *T. lawrencei* stocks showed marked differences from the *T. parva* stock profiles. Moreover, protein profiles were found to differ among *T. parva* Marikebuni or *T. parva* Muguga infected cell lines, originating from stabilates differing by one tick passage. Molecular markers using Tpr probes (TpM-23 and TpM-58) revealed similar profiles for Mariakani and Uganda, suggesting that they could be related (Conrad *et al.,* 1989; Chen *et al.,* 1991), whereas identical profiles were found for Muguga and Kilifi (Allsopp *et al.,* 1989). This might all point to a limited number of largely different stocks in the field and a good degree of relatedness amongst different stocks, but how this relates to cross-immunity is still a highly speculative issue.

What follows is an attempt to classify stocks according to existing cross-immunity data although often from experiments using a only few animals. This is by no means a true picture of differences in cross-immunity profiles but it is felt that clear breakthrough reactions might provide a basis for investigation of correlations between sequence data and immunity profiles related to these stocks. There is evidence that large differences exist in cross-protection profiles. The isolates shown in Table I can be grouped into four clusters, according to their cross protection characteristics in challenge experiments. The stocks are then further classified in an hierarchical order, with the arrow indicating the breakthrough direction. The top group contains stocks causing breakthroughs in the lowerlying groups. Stocks can be classified according to shared mAbs profiles into four groups and the numbers in brackets after the stocks relate to this classification. The letters relate to the country of origin (RWA = Rwanda, KEN = Kenya, UG = Uganda, TAN = Tanzania, MLW = Malawi, ZA = Zambia, ZIM = Zimbabwe and SA = South Africa). This classification is tentative as the position of some stocks has been derived from cross-protection shown by related stocks and not through direct challenge with these stocks. A final classification needs to be confirmed by additional cross-immunity experiments, using well-characterised stocks and defined stabilates, and significant numbers of animals per stock tested. There is no correlation found with specific mAbs profiles or the geographical origin of parasites.

Cluster A was formed by Marikebuni (4, KEN), Nyakizu (2, RWA), Uganda (2, UG) and Kasoba (?, MLW) and probably Mariakani (2, KEN).

Cluster B consisted of Chitongo (2, ZA), Kiambu5 (4, KEN).

Cluster C was formed by Boleni (1, ZIM), Lawrencei (?, KEN), Manyara (?, TAN), Pugu1 (?, TAN), Katete (2, ZA), and Mavueni (?, KEN)

Cluster D consisted of Muguga (5, KEN), Kilifi (5, KEN), Junju (?, KEN), Schoonspruit (5, SA) and Serengeti transformed(4, TAN)

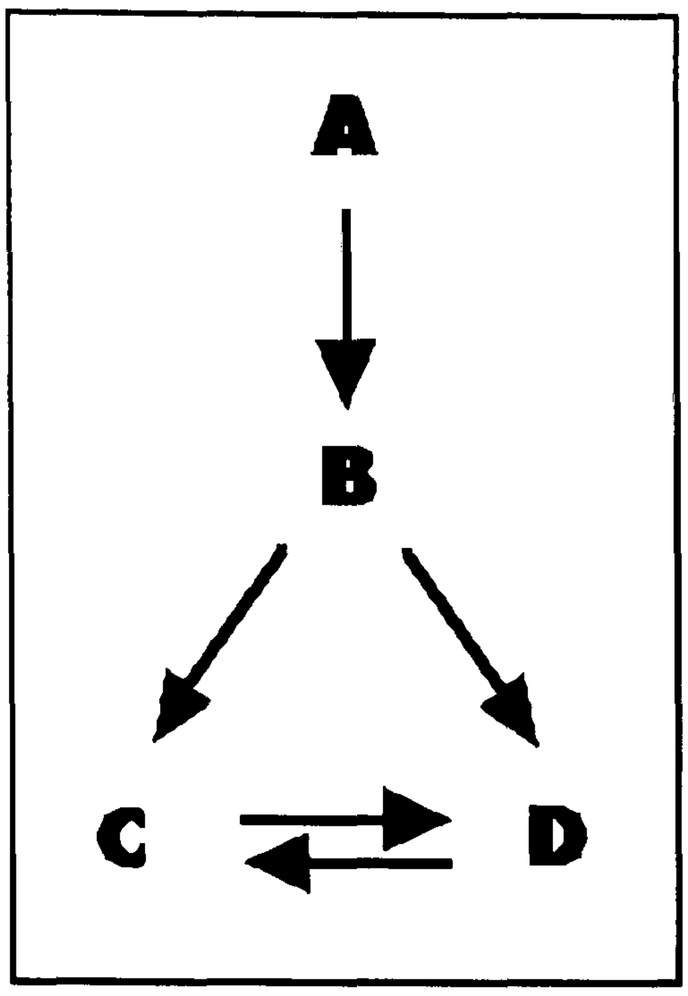


Fig. 4. Tentative classification of stocks according to their cross-immunity profiles, with the arrow indicating the breakthrough direction in protection. The top group contains stocks causing breakthroughs in the underlying groups.

Data from cross-immunity trials in which there were major breakthroughs show that these occurred in about 30% of the challenged cattle (Irvin *et al.*, 1983). This percentage is probably a reflection of the diversity in immune responses and biological limitations of the immune system due to natural variation in MHC. In the case of cross-immunity trials, it could be envisaged that a percentage of the animals mount immune responses which are less effective due to MHC- based differences in epitope affinity or slower response time (Musey, 1997). This could be true particularly as usually extreme challenge doses (multiples of the lethal dose) are used in very susceptible exotic breeds. Induction of immunity and degree of protection in *T. parva* have been related to thresholds in which increasing doses of sporozoites seem to be correlated with a more effective immunity (Cunningham *et al.*, 1974). It has been the rule in stabilate preparation to use ticks harvested during the peak of the parasitaemia to guarantee highly infected ticks (Young *et al.*, 1996). It can be speculated that such a stabilate production procedure might benefit parasite populations of mixed virulence, complying also with the concern to provide as broad a protection as possible by including all the antigenetically different populations present in a given stock.

A feature of most stocks from the southern part of the ECF range seems to be a low maximum piroplasm parasitaemia (Nyakizu, Uilenberg, 1982 and own data; Chitongo, own data; Boleni, Uilenberg, 1982 and *T. lawrencei*, Hill and Matson, 1970), in most cases, accompanied by a serious depression of the haematocrit.

Data have been difficult to compare as these influences might vary with sporozoite dose and host breed. It has been shown that different epidemiological situations might impose different selection pressures on parasites affecting their survival and transmission (Ewald, 1994). It could be envisaged that in an East African situation fast growing *Theileria* parasites might be selected as vectors are readily available throughout the year giving ample opportunities for the parasites to be ingested during the early stages of parasitaemia. These parasites would evolve towards fast multiplication in a race with the immune responses, perhaps ending in high pathogenicity and fast killing of the host. The parasites responsible for the carrier state would be at a disadvantage and eventually been lost from the field population. In contrast, the southern parasites would evolve towards slower multiplication rates as opportunities for transmission are restricted. Under these conditions, parasites with characteristics for induction of a carrier state would be at an advantage as prolonged presence in the host is crucial for their survival. This implies survival of the host and would certainly reveal major changes in the dynamics of parasite-host interactions. It could be argued that these stocks have evolved towards a lower propagation rate in the host leaving more time to mount good immune responses. This could explain the increased protection found with these stocks. It could also explain the depression of haematocrit through differences in accompanying cytokine and interferon profiles, either through changes in host response dynamics or differences in parasite induced signals. The author's data from stocks from Zambia support this hypothesis. Endemically unstable epidemiological conditions prevail in the Eastern Province of Zambia and these stocks show a parasitaemia in the range of 10-20%, whereas epidemic conditions in the Southern Province have given rise to stocks with lower parasitaemia (never over 6%) and a milder pathogenicity as is predicted. Analysis of data from 26 infections with Zambian stocks showed a marked drop in PCV of 50% starting on day nine and falling over a ten day period. White blood cell counts decreased to 50% from day seven over a ten day period and ended in either a terminal leucocytopaenia or a slow increase to normal values in the case of Eastern Province stocks. A transient phase of leucopaenia is followed by a second drop of the same magnitude with fast

increase to normal values in the case of high dose, or a slow increase in the case of a lower dose used with the Chitongo stock. Under the above hypothesis it could be expected to find parasites in Zambia with distinct phenotypes as the ecological conditions prevailing in this region would allow for different evolution. There is also some controversy about the mild nature of these stocks. Some *T. parva* stocks have induced mild reactions in Zebu cattle, although more severe reactions were observed in *Bos taurus* (Brocklesby and Bailey, 1968; Irvin *et al.,* 1989). Local isolates from the Southern Province showed mild disease and low parasitaemia (Musisi *et al.,* 1986; own data), although it is not known if this low virulence is a stable stock characteristic (see virulence and transmission relationship under 1.1.7.). Virulence (transformation) studies could aim at segregating the slow and fast reproducing parasite subpopulations for further study. Experiments have been initiated to test this hypothesis.

Uilenberg *et al.* (1982) reported the rapid passage in cattle of the *T. parva* Boleni stock (inducing lethal reactions in *Bos taurus* cattle) without affecting the low parasitaemia after seven passages.

Koch *et al.* (1988) reported a *T. parva* Boleni passage line inducing mild reactions but no markers were available at that time to study the mechanisms involved in the loss of virulence. An earlier Boleni passage has been used succesfully without tetracycline in immunisation in Zimbabwe (Kanhai *et al.,* 1997). Results of the DNA characterisation (using p67 marker, see under results) of Boleni isolates indicate the presence of at least two different components which might explain these inconsistent data.

Another argument in favor of this hypothesis comes from the observation that stabilates derived from the Boleni and Chitongo stocks have a wide immunogenic dose range (0.01-10ml in Koch *et al.,* 1988; own data on the Chitongo stock) and a higher LD50 than classical *T. parva* stabilates made from East African isolates. On the other hand difficulties have been experienced by different groups in producing highly concentrated stabilates from such stocks, although high infection rates in ticks are the rule, at least in the case of the Chitongo stock (own observation). It has been shown that *T. parva* Boleni does not induce high infection rates in eastern African ticks (Ochanda *et al.,* 1998),

reflecting the importance of tick stocks used for stabilate preparations. Parasite-vector interactions could play an important role in transmission efficiency (Ochanda *et al.,* 1998; Tempia *et al.,* 1997) up to a level where ticks might experience deleterious effects. It was found that highly infected ticks (with 100% of the salivary glands infected) were experiencing high mortality during moulting and reduced engorgement. This has been reported for *Babesia bovis* parasites in *Boophilus* ticks (Dalgliesh *et al.,* 1981; Agbede *et al.,* 1984) but the relevance of such studies to the field situation needs to be determined. Although information is available from stabilate production data (Young *et al.,* 1996) related to the eastern African situation, it is only recently that the importance of tick stocks in transmission has been considered. This shows the difficulties in phenotypic characterisation of parasite stocks when dealing with vector-borne parasites and the need for standardisation of reporting methods, including the origin of ticks used.

These interactions reported between the initial dose of sporozoites, cattle breed and differences in immune responses between individual animals will influence the dynamics of the host-parasite interactions and contribute to the pathogenesis observed. The outcome of these interactions could account for the variable results observed within certain stocks and shows the difficulty in dissecting the effects of the different interactions.

Irvin *et al.* (1983) reported that challenge results were not reciprocal. The issue of the carrier state following ITM immunisation has been a big concern to national veterinary departments. There was a genuine fear of introducing foreign strains into the local parasite population, if a carrier state was indeed present (Dolan, 1987). It was shown unequivocally by Dolan (1986a) and Young *et al.* (1986) that *T. parva* infections or immunisations resulted in carrier states, and later it was shown that these could be detected for up to 30 months using PCR amplification techniques (Bishop *et al.,* 1992).

A consistent feature of challenge trials has been the poor cross-immunity of cocktail immunised animals against *T. lawrencei* stocks (Radley *et al.,* 1979). In general, there is poor protection with cattle-derived parasites against buffalo-derived parasites.

1.2. Diversity in parasitic protozoa

The co-evolution theory and Red Queen hypothesis derived from it (Van Valen, 1973) have been fundamental to the understanding of parasite diversity and dynamics. Essentially, the close relationship between the parasite and its host (vector) implies that the parasite evolves in parallel with its host. This process of co-evolution has been pictured as an arms race between the host and the parasite (Red Queen Theory), whereby the host tries to mount more effective defenses while the parasite evolves more sophisticated evasion mechanisms. There is increasing evidence from the field in support of this hypothesis (Hill *et al.*, 1991; Thursz *et al.*, 1995). To be successful in such a dynamic process, both actors call on an array of mechanisms to generate diversity. Several pathogens have evolved efficient mechanisms to evade immune pressure.

1.2.1. Diversity generating mechanisms

The development of molecular techniques over the last decade has allowed extensive studies of the mechanisms underlying genetic diversity of protozoa. The *Plasmodium* genome has attracted major research effort as the genus comprises parasites of major importance to human health. *Plasmodium* spp are well known for their adaptability and versatility, and because some species are aetiological agents of malaria in laboratory animals, they have greatly facilitated research. Studies of different *Plasmodium* species have generated data against which results from *Theileria* investigations can be compared. The genome organisation of many protozoan parasites has a general common feature in that chromosomes show compartmentalisation into conserved central domains and variable ends. They show high frequencies of chromosomal rearrangements during meiosis and, to a lesser extent, during mitosis due in part to the presence of repetitive sequences at subtelomeric and telomeric regions. This facilitates homologous and heterologous pairing, and crossing-over events (reviewed by Lanzer *et al.*, 1995). Genome plasticity in *Plasmodium* is characterised by frequent loss of genes which become redundant under changed conditions *in vitro*. Moreover, extensive polymorphism in subtelomeric repetitive regions as well as rapid and apparently concerted variation in the

intragenic repetitive arrays that are typical of plasmodial antigen genes, all contribute to this plasticity. Frontali (1994) showed that repeat homogenization mechanisms in *Plasmodium*, acting at the DNA level, could lead to rapid fixation of variant epitopes and appear to be an important mechanism in the evasion of the immune defenses in the vertebrate host.

Antigenic variation is a mechanism used by parasites whereby they switch the expression of a particular protein using different alleles of a gene. These different genes make up a gene family in many parasitic protozoa and other organisms (*Plasmodium, Trypanosoma, Giardia, Borrelia, Pneumocystis*) that deploy antigenic variation as a mechanism to evade host responses. These mechanisms are based on DNA rearrangements, effecting changes in serial gene expression in the case of *Trypanosoma brucei*. This parasite possesses a stock of more than 1000 variable surface glycoprotein (VSG) genes. This mechanism may also contribute to the diversification of the array of already existing VSG sequences by generating new sequences. The control mechanisms of antigenic variation in *Giardia* or *Plasmodium*, both possessing a similar repertoire of variable surface protein (VSP) or variable (var) genes, have not been elucidated and might be different.

1.2.2. Basis of genomic variability

The total genomic Guanine/Cytosine content (G/C content) in *P. falciparum* is very low (19% overall) with Adenine/Thymine -rich (AT-rich) coding (70%) and non-coding (90%) regions. Although condensed chromosomes have never been observed, the haploid number has been resolved by PFGE to be 14 (Walliker, 1989). The *T. parva* genome has a total genomic GC-content of 30% (Allsopp and Allsopp, 1988) and the telomeres consist of 38% GC-rich codons. This is in marked contrast to a normally inverse correlation between telomeric and total genomic GC-contents in most organisms (Petracec *et al.*, 1990).
It has been argued that high AT-rich genomes might facilitate strand displacement during periods of rapid DNA replication that occurs during gametogenesis (Janse *et al.*, 1986 and1988). A high AT-content results in a

relative weaker bond between the DNA strands and this might be correlated with high adaptive value by contributing to chromosome instability and polymorphism through breakage and telomere healing (Pologe and Ravetch, 1988; Scherf *et al.*, 1992). On the other hand, it will compromise the parasite in its choice of ideal amino-acids at crucial sites in a particular protein (Bernardi and Bernardi, 1986). But whatever the reason for this AT-rich use, it must confer considerable selective advantage (Musto *et al.*, 1995 and 1997).

Another important diversity generating mechanism in *Plasmodium* spp is embedded in the 100kb telomeric associated sequence (TAS) region. This region consists of blocks of tandemly repeated sequences located between 10kb and 45kb upstream from most telomeres, and these sequences are thought to be involved in the generation of polymorphism through DNA rearrangements (reviewed by Janse, 1993).

The subtelomere structure in *T. parva* is different. Sohanpal *et al.* (1995) found 500-1000bp long telomeres with the lowest percentage (37.5%) of GC-rich sequences known, separated from the TAS region by a 126 bp conserved region. The TAS region was also AT-rich (70%) and had only 2 x 22bp long repeat sequences, a different subtelomeric make-up from *P. falciparum*. The genome of *T. parva*, and of *Babesia* spp are organised differently, with only four chromosomes of similar sizes (Morzaria and Young, 1992). In *T. parva*, the repetitive sequences (Tpr), located in the centromeric region of chromosome three, are most intriguing. These sequences are interspersed with short regions of unique sequence and comprising 1.3% of the total genome (Baylis *et al.*, 1991, Bishop *et al.*, 1997). In most protozoa such variable regions have been located in the telomeric regions (reviewed by Lanzer *et al.*, 1995).

1.2.2.1. Variability of subtelomere domains

Frequent expansion and contraction of repetitive subtelomeric sequences resulting from sister chromatid interactions, recombination between heterologous chromosomes during mitosis or slippage of the DNA polymerase during replication are all common mechanisms that have been shown to operate in diversity generation in the protozoa (Corcoran *et al.*, 1986; Lanzer *et*

al., 1993). The underlying basis has been attributed to the special make-up of the subtelomeric region which facilitate heteroduplex formation and interaction between strands.

1.2.2.2. Truncations

Truncations have been shown to occur spontaneously in *Plasmodium* during mitosis, mostly under *in vitro* cultivation conditions. They are initiated by double strand breaks and *de novo* synthesis of telomere repetitive sequences at the breakpoint. Several hotspots for double strand chromosome breakages have been identified. The hotspots found in the KAHRP gene correlate with nucleosome repeat units. Lanzer *et al.* (1994) suggested that at the intersection of two consecutive nucleosome units, the DNA sequence might be more exposed to external influences, resulting in breakage. It is thought that truncations could play a role in the sequential gene expression from multicopy polymorphic genes (var genes) stored in the genome. This antigenic variation could either be driven by DNA rearrangements, affecting changes in gene expression or due to other mechanisms yet to be defined.

1.2.2.3. Recombinations

The genetic connotation of the term recombination means reshuffling of genes due to separate segregation of the parent chromosomes after meiosis. The term used here in the context of molecular genetics refers to the recombination between chromosome regions as a result of crossing-over and gene conversion events. It has been shown that complete sequence homology over a DNA stretch of 24-74 bp is sufficient for genetic recombination (Singer *et al.*, 1982). Genetic recombination may be the result of a crossing-over with reciprocal or non-reciprocal exchange between chromosome parts.

- Chromosomal crossing-over with reciprocal exchange of a chromosomal part between homologous chromosomes is a very common mechanism, affecting the linkage between alleles.
- A special crossing-over is an intragenic recombination involving a crossing-over event within the coding region of a gene.

- Classical gene conversion in which one allele is converted to its sister allele during meiosis is a mechanism frequently observed in fungi.

Recombination frequencies have been extensively studied in yeast. Meiotic crossing-over is a frequent phenomenon, while crossing-over during mitosis occurs a 1000 fold less frequently.

It has been suggested that frequent intragenic recombination leading to the characteristic mosaic structure of some immunogenic genes (MSP genes in *Plasmodium*) could be an alternative strategy generating diversity in protozoa.

1.2.3. Genetic polymorphism

Three main theories are put forward to explain the generation and maintenance of genetic variation within and between species. These theories are known as the selection, the neutral and the molecular drive theory. The importance attached to the different mechanisms in the generation of variability on which selection would act constitute the main difference between these theories. The selection theory was first put forward by Darwin who argued that only advantageous mutations will be selected. The evidence was based on phenotype characteristics reflecting the knowledge at that time. The neutral or the closely related nearly neutral theory (Kimura, 1968; Otha, 1973) stressed the importance of neutral mutations (without direct impact on the phenotype) and genetic drift as the main forces leading to diversity. This theory has its basis in the observation that non-coding sequences are much more diverse than coding sequences. The neo-Darwinian theory accommodates the modern insights into molecular genetic structure and has resulted in considering genetic variability generated by different forces independently of selection but as a prerequisite for it. The recent molecular drive theory identifies the genomic processes of turnover as a major force (Tautz *et al.*, 1986) and an increasing amount of data support this. The molecular drive theory is strengthened by the fact that subsets of genes have been found which are notably prone to mutations. The mechanisms affecting a small part of the genome are somatic mutations involving single (or double) nucleotide substitution and gene conversions requiring a template. Various mechanisms have been identified which generate more substantial

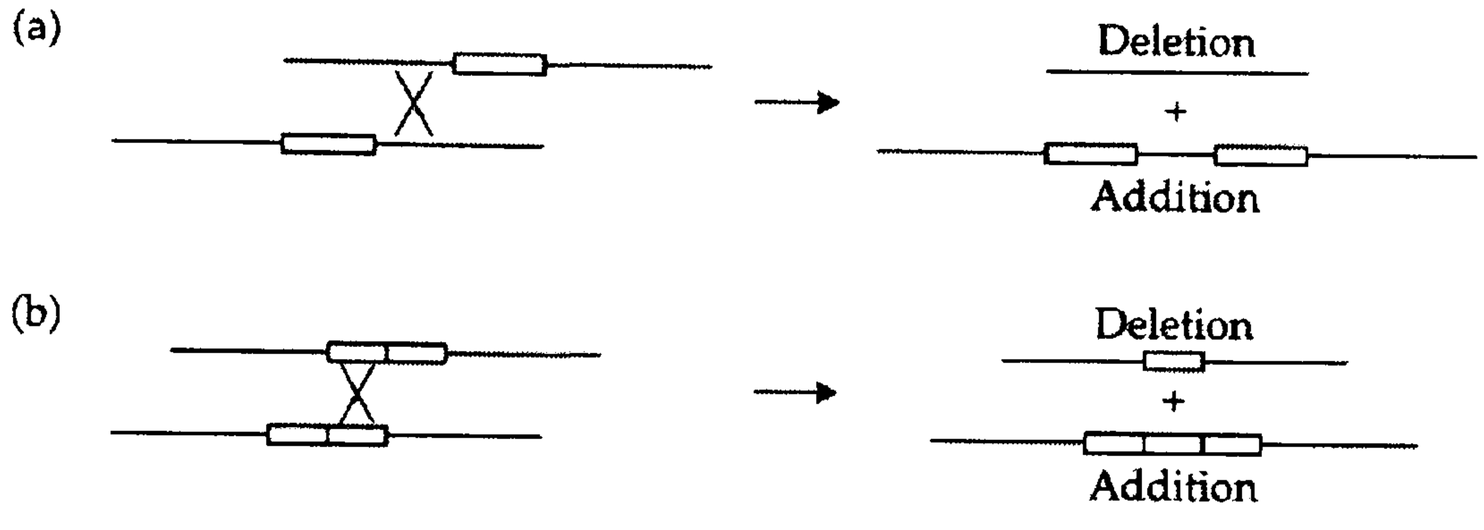
modifications. These are transposition of mobile elements, called transposons (Charlesworth, 1996), to other regions of the genome, slipped strand mispairings, gene rearrangments and gene conversions. It has been argued by Kimura (1969) and Otha and Kimura (1973) that these mechanisms tend to minimize deleterious effects on fitness thereby promoting these in the generation of new sequences.

1.2.3.1. Hotspot regions of high frequency insertions/deletions

In yeasts and all eukaryotes, large regions of heteroduplex DNA (synaptonemal complexes) are formed between homologous chromosomes during meiosis, leading to an optional appearance of recombination nodules (Carpenter, 1994a). Some of these nodules result in genetic crossing-over events while others appear to be associated with the occurrence of gene conversion (Carpenter, 1994b). Several mechanisms have been implicated in the origin of hotspot regions.

- a. Unequal crossing-over, based on alignment problems due to long repeats is one mechanism causing large insertions or deletions, especially if long tandem repeats are present. Unequal crossing-over creates a sequence duplication in one chromosome and a corresponding deletion in the other. It may occur between two sister chromatids (1%) or between two homologous chromosomes at meiosis (17%). The intensive study of recombination mechanisms in *E.coli* has revealed specific sequences acting as a switch in the unwinding process, thereby downregulating its 3'-strand specific nuclease activity (Dixon and Kowalczykowski, 1993) and promoting recombination through the production of a single strand DNA fragment. This mechanism has been mimicked in human cells in culture by using a consensus sequence based on the human hypervariable minisatellite suspected to be involved in promoting recombination (Wahls *et al.,* 1990). The specific sequences has been found to be composed of 5'-GCTGGTGG-3' in *E.coli* (Smith *et al.*, 1981), and suspected to be 5'-GGAGGTAGGCAGGCAG-3' in the mouse

MHC recombination hotspot, 5'-GTGACTGGCCAGGAGG-3' in the hamster aprt insertion/deletion locus and 5'-GGGCAGGAXG-3' in human minisatellites (Jeffreys *et al.,* 1985). A related sequence has been found in the ade8 gene of *Saccharomyces cerevisiae* (White *et al.,* 1988). They are all characterised by a high GC content. The result is a site specific recombination event involving recognition and cutting of heptamer-nonamer recombination signal sequences flanking the rearranging elements.



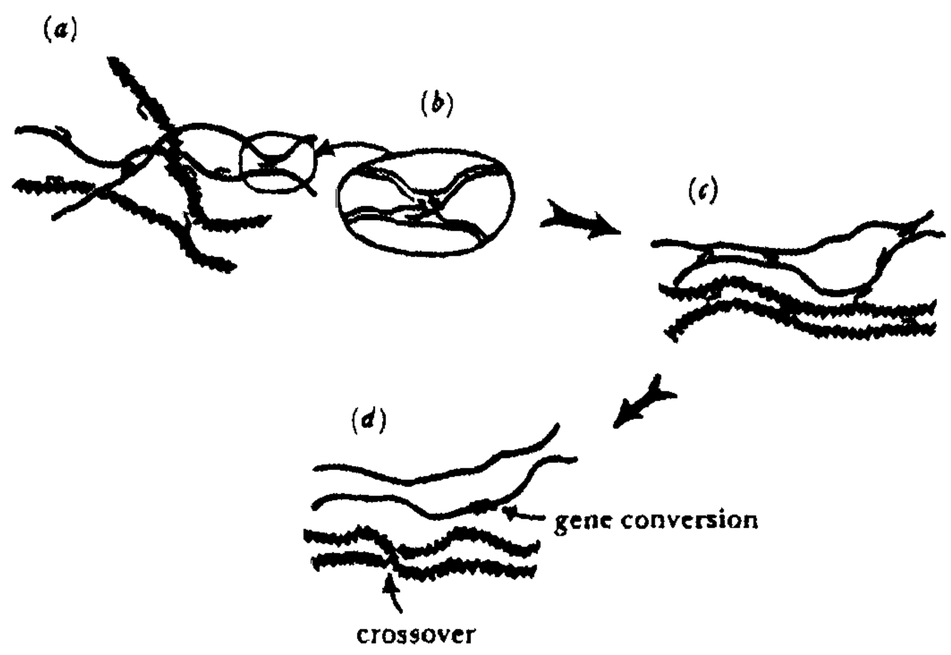
Unequal crossing-over. A box denotes a particular stretch of DNA. When a DNA segment is duplicated in tandem as in (b), the chance of misalignment increases and so does the chance of unequal crossing-over.

Fig.5 Unequal crossing-over (from Li, 1997)

- b. Slipped-strand mispairing is another mechanism and is more frequent in cases of short repeats and palindromes, giving smaller insertions or deletions of 20-30 bp differences. It has been argued that so called cryptic simplicity in DNA is a major source of genetic variation (Tautz *et al.,* 1986). Cryptic simplicity is defined in DNA as the universal existence of regions of single or few short, usually tandemly repeated sequence motifs playing a role in gene conversion, recombination, telomere replication signals or gene regulation. Slippage has been proposed as the most reasonable mechanism, generating and using these simple short motifs (Tautz *et al.,* 1986).

- c. Gene conversion as used here refers to a recombination event over a short region between alleles or loci, involving parts of genes as at the human class-II loci (reviewed by Erlich and Gyllensten, 1991). Classical gene

conversions involves the biased or unbiased exchange between two alleles on homologous chromosomes and has a frequency range between 0.5%-18%. There is conclusive evidence that gene conversion events can also affect regions in the order of tens of nucleotides (Smithies and Powers, 1986) than the usual larger (> 250 bp) areas



An illustration of the proposed relation between chromosomal pairing and gene conversions. Two homologous pairs of chromatids, represented by straight lines and zig-zag lines, are shown. (*a*) Single-stranded DNA 'feelers' are extruded at many positions along the unsynapsed chromatids and invade nearby DNA molecules. (*b*) An enlarged view of a typical extrusion and invasion. The single-stranded feeler, extruded from the upper chromatid by newly synthesized DNA shown as a dashed line, invades the lower chromatid. (*c*) 'Scanning' leads to some stable invasions. 'Zipping' of the homologous chromosomes, complete for the zig-zag and partial for the straight chromatids, has occurred because of cooperation between nearby invasions. (*d*) Once the homologous chromatids have paired, the machinery permitting crossing over can be set in motion. Stable invasions between non-homologues will not be sufficiently frequent to permit the zipping effect. In these cases, the 'footprints' of the invading feelers may occasionally lead to gene conversions.

Fig. 6. Relation between chromosomal pairing and gene conversion. Picture from Smithies and Powers, 1986.

- d. DNA transpositions generate usually long insertions or deletions. They are the result of an insertion of a movable DNA region, called a transposable element (TE) and can be detected by small segments (4-12bp) of duplicated host DNA at the insertion site, giving a characteristic direct repeat (Li, 1997 p 336). Some TEs might show high preference for one specific genomic location. The shortest TEs found are in the range of 700bp.

- e. Double strand breaks are thought to play an important role in transpostions, translocations and certain gene conversion events like V(D)J-recombination of immunoglobulin genes (Weaver, 1996). It is through the specific repair mechanisms that point mutations or other polymorphism might be generated. Two repair mechanisms have been proposed: the homologeous recombination (HR) model (Szostak *et al.,* 1983), needing significant sequence homology and a DNA-end-joining model (Roth *et al.,* 1985).

In the HR model an endonuclease will induce a double strand break in a homologeous area of one of the two duplexes, followed by a 5' to 3'-exonuclease activity creating a large gap. This allows a 3' single strand (donor) to interfere and invade the other duplex (acceptor) and form a displacement loop (D-loop).

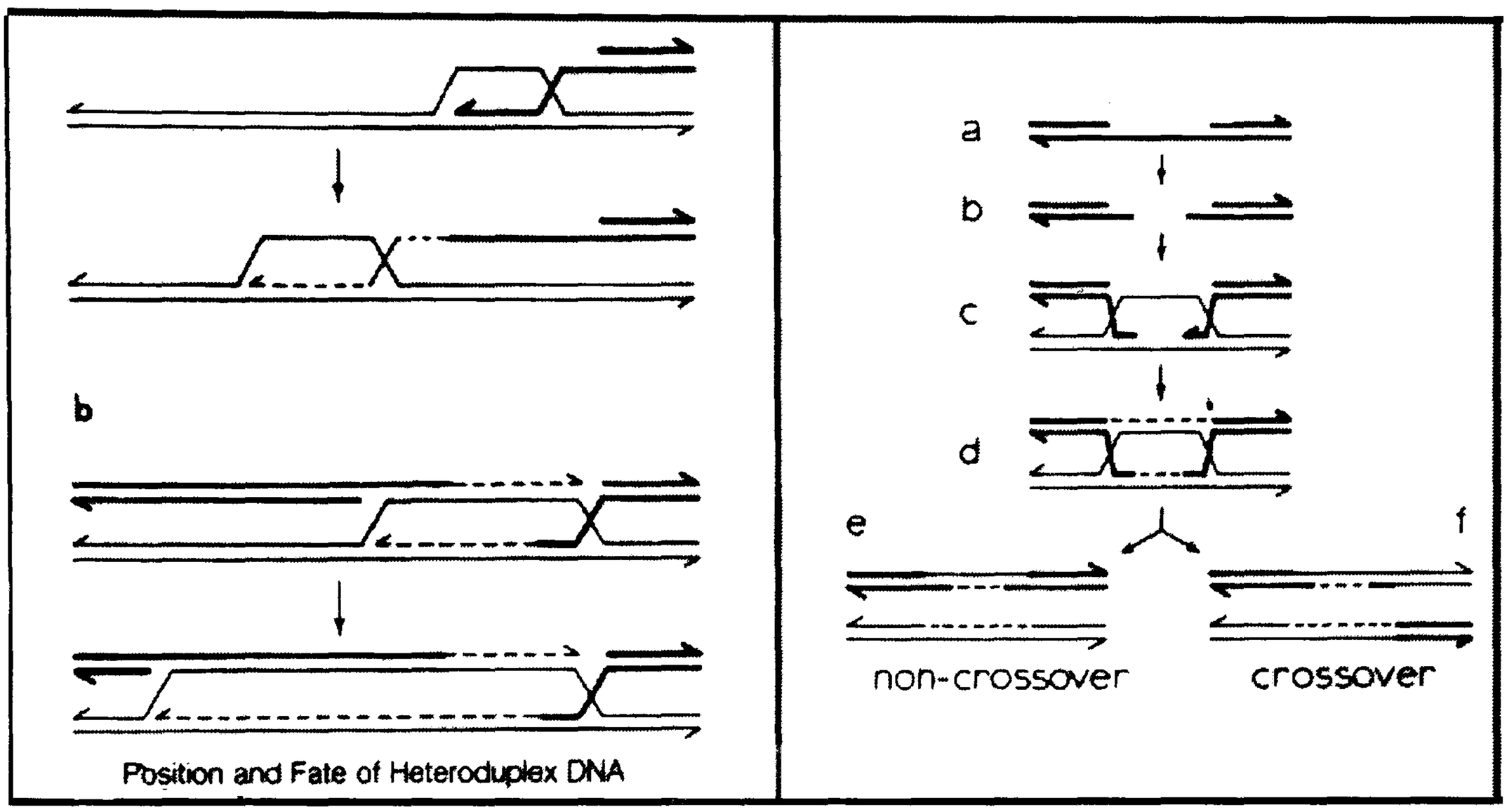


Fig.7. The HR model of double strand repair. Left picture shows heteroduplex DNA creation by strand invasion and annealing. Righ picture shows a double strand break repair model in which all asymmetric heteroduplex DNA is confined to one chromatid. Both pictures from Szostak *et al.*, 1983.

This is most likely orchestrated by RecA-protein-like enzymes as described in *E.coli* (Howard-Flanders and Theriot, 1966). The D-loop is enlarged by single strand repair activity, using the invaded 3'-end as primer. Once the majority of missing nucleotides are synthesised, the old displaced strand will migrate towards the remaining single strand due to complementarity and the same synthesis mechanism will repair the missing nucleotides on this strand. The gap will be filled completely by the donor sequence. This leaves two chiasmata that will be dissolved via small break and repair mechanisms, involving endo- and exonucleases. This repair system is based on long homologous sequences which occur mostly in coding regions of the DNA. The genome of lower eukaryotes consists mainly of coding areas and the HR model might be sufficient to handle or repair jobs.

This system cannot deal with all repair work in higher eukaryotes as there is lots of non-coding DNA, characterised by the frequent presence of short repeated sequences. Therefore the DNA-end-joining mechanism would be able to repair regions where long homologous sequences are absent. The single strand annealing (SSA) model covers a repair mechanism based on the helicase activity to unwind a duplex (annealing between nearby short repeat sequences with synthesis and rejoining of the new sequences). This leaves point mutations and small to large deletions or insertions. The size of the latter depends on the position of the short tandem repeats in relation to the double strand break.

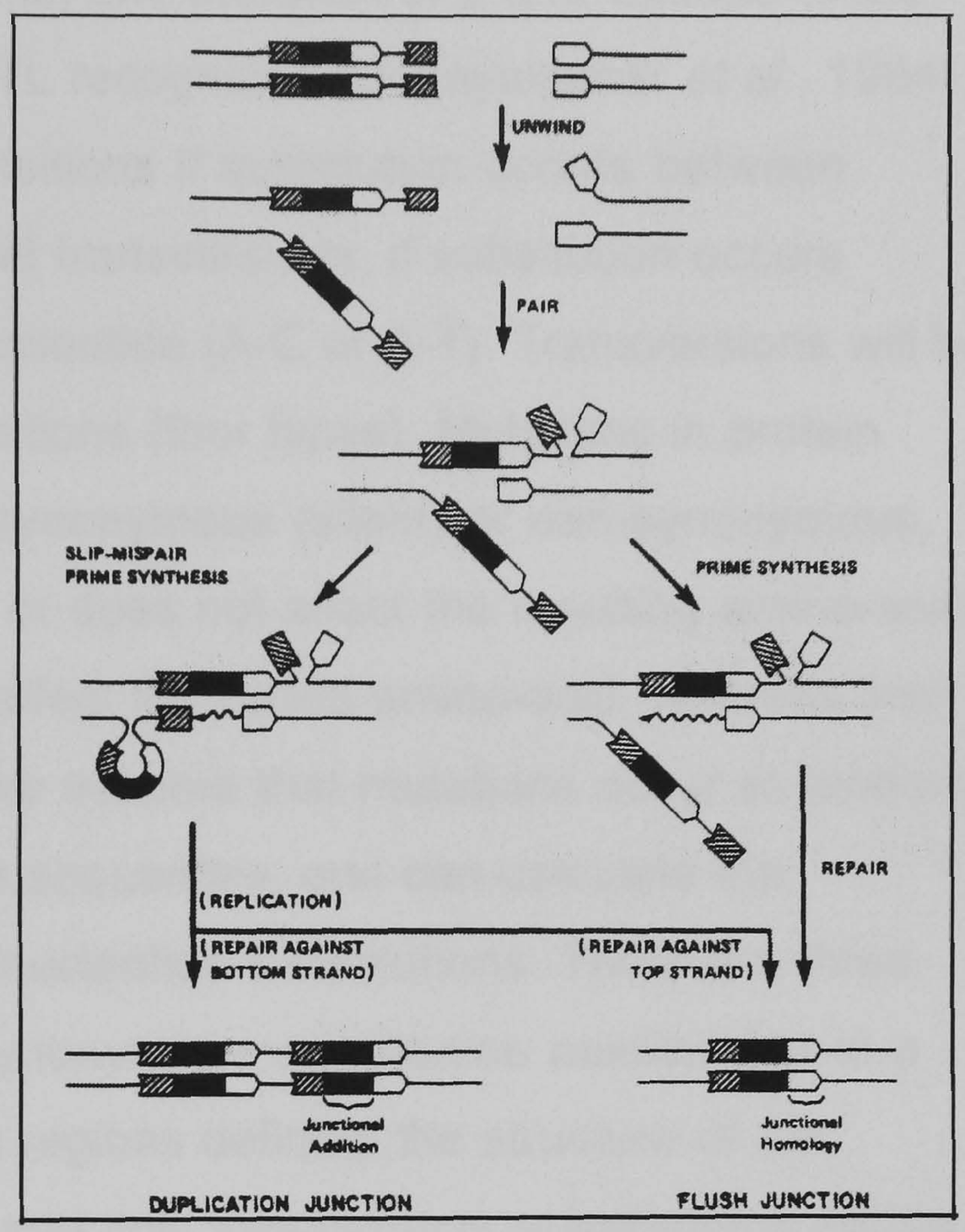


Fig. 8. DNA-end-joining repair mechanism for non-homologous recombination. Short homologies (open arrowheads) are uncovered through which duplexes can transiently pair. Picture from Roth *et al.,* 1985

It is not known which of these mechanisms is responsible for the occurrence of hotspot recombination or if particular enzymes promote specific mechanisms. But it becomes more and more clear that powerful mechanisms are active during meiosis, based on ubiquitus repair mechanisms and driving extensive genome and gene variability in eukaryotes. This can be extended to *T. parva* parasites, although information on chromosome structures and replication mechanism is far from complete. The available data on the closely related *Plasmodium* species indicate that they resemble eukaryotes in many aspects (Sinden and Hartley, 1985; Kemp *et al.,* 1990), including eukaryote-like DNA polymerases (Fox *et al.,* 1991; Ridley *et al.,* 1991).

1.2.3.2. Point mutations

Point mutations in immunodominant epitopes are an important mechanism of immune evasion. It has been shown that one mutation in a CTL epitope of the *Plasmodium* CS protein abrogates CTL recognition (Udhayakumar *et al.,* 1994). Point mutations are classified as transitions if subsitution occurs between purines (A-G) or pyrimidines (C-T) and transversions, if subsitution occurs between a purine and a pyrimidine nucleotide (A-C or A-T). Transversions will be more frequent (eight types) than transitions (four types). Mutations in protein coding regions can be classified as synonymous (silent) or non-synonymous, depending if the codon change does or does not affect the resulting amino-acid. Most third position mutations do not affect the coded amino-acid, whereas first and second position changes do. If we assume that mutations occur at random and that there is no codon bias in the sequences, one can calculate the expected proportions of the different nucleotide substitutions. There are three times more non-synonymous than synonymous substitution possibilities in a sequence. In a coding sequence, the regions defining the structure of the peptides are usually very conserved and only synonymous substitutions will be found, whereas antigenic epitopes are not constrained to the same extent and non-synonymous substitutions will occur as well. Non-synonymous changes have been considered as a sign of selective pressure for nucleotide sequence divergence leading to evasion of host immune responses. The rate of nucleotide changes differs between genes and even gene regions, and synonymous substitutions occur more frequently than non-synonymous alterations. Regions with high mutation rates are called hotspots. Several specific characteristics have been defined and one of them is based on the -CG- sequence, where cytosine is frequently methylated in higher eukaryotes, giving error prone replication. The presence of palindromes contribute to higher mutation rates and short tandem repeats are often hotspots for deletions or insertions (Li, 1997). Mutations within a gene have been shown to increase the rate of meiotic gene conversion and crossing-over but this seems often to be context-dependent (Ponticelli *et al.,* 1988).

The DNA error rate per base multiplication is in the order of 10^{-9}. The mutation rate in eukaryotes producing visible or lethal effects per gene locus (mean of 600bp) per cell division has been estimated between 10^{-6} and 10^{-7}. The rate of slightly deleterious mutations is 20 times higher than the rate of lethal mutations. The rate at which new phenotypic variation is generated by mutation has been estimated at 0.1% per generation (Maynard Smith, 1998).

1.2.3.3. Concerted evolution

Concerted evolution is a homogenizing process initially observed in satellite DNA sequences responsible for rapid divergence between homologous sequences in related species (Brutlag, 1980). Tandemly repeated elements in eukaryotic genomes often show a high level of conservation within a given species in contrast with their rapid interspecific diversification. This has also been suggested in the case of homogenisation of repeated sequences in *Plasmodium* genes (Arnot *et al.,* 1988; Frontali and Pizzi, 1991). The underlying mechanisms were described by Dover (1982) in his molecular drive model based on mechanisms of unequal crossing-over, slipped replication and gene conversion as the cause of homogenization in tandemly repeated sequences during meiosis. Biased classical conversion involves the correction of mismatches in favor of one of the input strands after misalignement of repeats.

1.2.3.4. Var Gene families

Members of widely different genera have evolved an immune escape mechanism called antigenic variation based on the presence of a family of variable genes scattered more or less over the entire genome. Antigenic variation has many manifestations, variable antigen (VSG) in *Trypanosoma* (reviewed by Borst and Greaves, 1987), variable major proteins (VMPs) in *Borrelia* (Saint Girons and Barbour, 1991), variable surface proteins (VSP) in *Giardia* (reviewed by Nash, 1995), var genes in *Plasmodia* (reviewed by Berendt *et al.,* 1994) and the major surface glycoprotein genes in *Pneumocystis*. These genes code for important parasite surface proteins and are expressed in alternation at a low and fixed rate per generation so that the immune system of

the host does not recognise the different populations. The overall structure of the genome of these parasites has evolved to allow and promote DNA rearrangements instrumental in the generation of these complex genes and control of their expression (reviewed by Janse, 1993; Lanzer *et al.,* 1995). The molecular mechanisms of differential expression have been unraveled for *Trypanosoma* (Pays *et al.,* 1981; Kooter *et al.,* 1987) but not yet for the others.

1.3. Diversity in Theileria and the related protozoan Plasmodium

Although there is no indication that antigenic variation would be an important mechanism in *T. parva*, genome diversity has been extensively studied in *Plasmodium* and the variety of mechanisms used by this parasite was thought to form an important basis from which to explore the polymorphism found in the related *T. parva* parasite.

The term genome diversity is used to describe variability between two organisms reflected by differences in genome-wide analysis results, including differences in gene locations. The term gene diversity is used to describe polymorphism between two organisms at the gene level.

1.3.1. Genome diversity in *Plasmodium*

It is thought that the diversity is exhibited by most parasitic protozoa in order to compete effectively in the arms race with host immune responses. A flexible genome would better guarantee their survival during the complex interactions in the different cellular environments in vector and host, as most parasitic protozoa have evolved complex life cycles. The genomic flexibility in *Plasmodium* is manifest by the size variability between homologous chromosomes (up to 25% of chromosome length) of various isolates (Kemp *et al.,* 1985; Van der Ploeg *et al.,* 1985) and in Southern blot profiles (reviewed by Janse, 1993). The genome consist of 3×10^7 bp (Weber, 1988) organised in 14 chromosomes ranging in size from 600 Kb to 3.5 Mb and an extreme high AT-content of 81% (Wellems *et al.,* 1987; Kemp *et al.,* 1987). The mechanisms generating karyotype diversity are multiple, including translocations and recombinations, loss of telomeric sequences by breakage and telomere formation on the broken ends. It has been

shown that size differences arise during meiosis as well as during mitotic multiplication through DNA rearrangements, mainly localised in the subtelomeric regions (Vernick *et al.,* 1988). The telomeric model of diversity generation has become a paradigm in malaria (reviewed by Janse, 1993 and Lanzer *et al.,* 1995). The subtelomeric region in *P. falciparum* chromosomes spans a rather conserved region of 40kb, encompassing the Rep20 region. The latter consists of tandemly arranged 21bp long imperfect repeats (Pace *et al.,* 1995). Subtelomeric regions (50-250kb long) often contain non-coding repeat sequences which frequently experience deletions and chromosome breakage (Hinterberg *et al.,* 1994). Genes coding for a number of immunodominant parasite proteins are often located in these fragile chromosomal segments (Harrington and Greider, 1991; Morin, 1991). These data suggest that the compartmentalisation of *P. falciparum* antigen genes towards the chromosome ends is related to their expression and leads to gene families scattered on several chromosome extremities. This has been confirmed in a number of studies (Rubio *et al.,* 1996; Hernandez-Rivas *et al.,* 1996 and 1997; Scherf *et al.,* 1996) and seems to be a specific mechanism of genome adaptation by *P. falciparum* to its host environment, although similar mechanisms have evolved in a number of other organisms (Pryde *et al.,* 1997).

This remarkable genome structure is especially prominent in the case of chromosome nine, which shows size polymorphism correlated with pathology reflected in the *in vitro* loss of cytoadherence capacity. The gene coding for cytoadherence is lost through the action of a deletion. The chromosome contains a unique ORF sequence in the genome (the breakpoint open reading frame, BPORF) shown to be a hotspot for breakpoints (Bourke *et al.,* 1996; Lanzer *et al.,* 1994b). This hotspot has been related to the chromatin structure and might be common in other eukaryotes (Lanzer *et al.,* 1994). The biological relevance of these rearrangement mechanisms in wild type parasites is not yet clear.

1.3.2. Genome diversity in *Theileria parva*

The first successful attempts to characterise genomic diversity in *T. parva* isolates *in vitro* were based on the analysis of mAbs profiles of different stocks

(Pinder and Hewett, 1980; Minami *et al.,* 1983). Genomic characterisation of *T. parva* using isoenzyme analysis proved very difficult due to the intracellular location of the parasite during the schizont stage and the apparently low

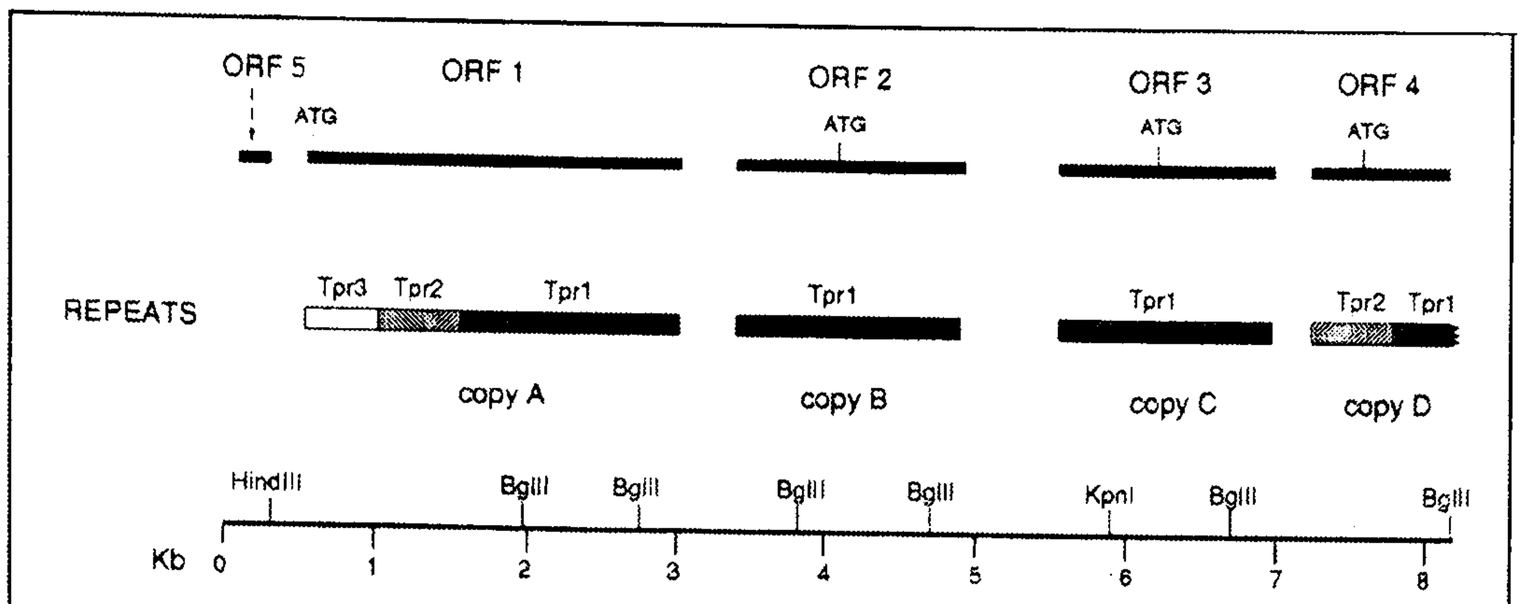


Fig. 3. Diagram showing features of the sequenced region of λ11.22. The position of the first in-frame ATG start codon is shown for ORFs 1–4 but not ORF 5. Only a selection of restriction sites are shown.

Fig. 9. Organisation of Tpr region in *T. parva*. Diagram from Baylis *et al.*, 1991.

metabolic activity of the bloodstage (Allsopp *et al.,* 1985). The basis for the molecular characterisation of the *T. parva* genome (totaling 10^7 bp) was laid as a result of the genome mapping activities at ILRI, Nairobi.

A physical map of large restriction fragments was constructed using the linking clone approach (Morzaria and Young, 1993). Four chromosomes of 3.2, 2.3, 2.2 and 2.1 megabases were detected by pulsed-field gel electrophoresis (PFGE) and confirmed by telomeric probing patterns. Moderate karyotype polymorphism was found between the 29 *Sfi*I fragments of different isolates, mostly on telomeric fragments (Morzaria *et al.,* 1990). Several DNA probes (IgTpM-23, M-112 and M-58) were generated which detected genomic diversity in *T. parva* stocks, using restricted fragment length polymorphism (RFLP) techniques (Conrad *et al.,* 1987) and discriminated among isolates showing identical mAbs profiles. The IgTpM-23 probe, based on a repetitive region, showed the best resolving power in discrimination among isolates. It was not clear if the results correlated with real genomic differences as variation in profiles was found between extracts derived from corresponding schizont and piroplasm material.

The IgTpM-23 probe was improved by Allsopp *et al.* (1989) using a 623bp fragment cloned in pUC8 (designated Tpr1) that gave good discrimination when tested on 17 different *T. parva* or *T. lawrencei* stocks. It was also shown that the Tpr1 sequence could vary within a clone. Further studies revealed extreme divergence of this multicopy locus (Baylis *et al.,* 1991; Bishop *et al.,* 1997) and it

was shown to consist of tandemly arrayed open reading frames (ORFs) containing various repeated elements (Tpr1, Tpr2 and Tpr3).

The Tpr locus is predicted to encode proteins that are expressed only in the piroplasm stage (Baylis *et al.,* 1991). The locus consists of multiple copies of three long repeat units within the large ORFs as well as isolate specific sequences. These repeat units show good homogeneity amongst different isolates, confirming the results of the Tpr1 based probe (approximately 100 copies per genome). Isolate specific regions are located at the 3' terminal ends of the ORFs and could be used as intra-specific marker (Bishop *et al.,* 1993a) It was suggested that concerted evolution could be the basis of this fast evolving polymorphism and might be a mechanism for the generation of novel genes (Bishop *et al.,* 1997). The large ORFs have been shown to be transcribed in the piroplasm but not in the schizont stage (Baylis *et al.,* 1991). They are present also in other *Theileria* species but no peptides corresponding to these sequences have been demonstrated.

A *P. berghei* based telomeric and a ssu rRNA ribosomal DNA probe have been added to the existing characterisation tools for *T. parva* and tested on 21 stocks originating from throughout the ECF distribution. The ribosomal probe could discriminate two groups while the telomeric probe, which can only be used on piroplasm DNA, separated eight isolates into four groups. Tpr1 was shown to be the most discriminatory probe, characterising 20 different genotypes (Bishop *et al.,* 1993b). It shows clearly the power of this characterisation method in revealing extensive genome polymorphism among *T. parva* stocks.
A *T. parva* telomeric probe (pTpUtel) was subsequently developed that could be used on DNA derived from schizont-infected lymphocytes (Bishop *et al.,* 1996). This probe showed consistent RFLP pattern from a cloned isolate after four tick/cattle passages. Furthermore, there are indications that the telomeric regions of *T. parva* may be relatively stable. Characterisation of the ends in two cloned telomere regions showed no significant repetitive sequences up to 16kb proximal of the telomeres and a lack of extensive sequence homologies with telomere regions of the other chromosomes (Sohanpal *et al.,* 1995). This is in

contrast with *Plasmodium* and suggests that rearrangements in telomeric repetitive DNA sequences are not the main mechanism leading to the observed *Sfi*I fragment polymorphism.

1.3.3. Gene diversity in *Plasmodium*

The diversity in *P. falciparum* has been studied widely in relation to vaccine development. Extensive polymorphism has been found at the gene level, especially in those antigenic genes, coding for membrane proteins, expressed during the bloodstage. The main diversity generating mechanisms in *Plasmodium* have been found to be based on point mutations, deletions/insertions and occasional intragenic recombination or gene conversion events.

Sequence comparison of various antigens has been important also in our understanding of the molecular basis of immune evasion mechanisms. The main assumption, formulated by De La Cruz *et al.* (1987) as the immune selection hypothesis, is based on the fact that changes in sequences will result in loss of recognition by cells sensitized to another sequence. Regions of the DNA sequence with a total lack of synonymous substitutions, indicative of strong selective pressure, have been identified in some important antigens. For the circumsporozoite (CS) protein, it has been shown that these correlated with regions known to be important in T-cell activation or shown to be potential targets (De La Cruz *et al.,* 1989; Lockyer *et al.,* 1989 and 1998). A description of the variability in *Plasmodium* genes showing homologies in function or structure with genes of *Theileria* provides a basis to search for parallels between the two organisms.

1.3.3.1. Gene structure of sporozoite stage antigens

The circumsporozoite (CS) protein covers the surface of the sporozoites of *Plasmodia* and is encoded by a single copy gene. It has a similar structure in all species, comprising of well conserved terminal regions coding for signal and anchor sequences flanking a central region of variable species-specific tandem repeats. The tetrapeptide sequences seem well conserved but variation in copy number causes minor size polymorphisms, even between clones of a single isolate. Sequence analysis of different isolates revealed the occurrence of

multiple unequal cross-over events, point mutations and deletions (Lockyer and Schwarz, 1986). There has been evidence of selective accumulation of point mutations within these immunosensitive sites, interfering with CTL recognition (Hill *et al.*, 1992; McConkey *et al.*, 1990). The preference of the AAC over the normal AAT codon for the second Asn in the repeat sequence is suggestive of strong selection pressures. The repeat region contains a B-cell epitope while CD4/CD8 determinants are situated in the C-terminal, genus conserved, region in *P. falciparum*. The same antigen has a similar structure in *P.vivax*. Arnot *et al.* (1988) studied diversity in several *P. vivax* isolates from Asia and Latin America. The gene sequence is identical, apart from the central domain and a 48 bp insert flanking the 3' end of the repeats. One tandem repeat consists of nine amino-acids, repeated 20 times and displaying good conservation among the repeats. Variations within the repeats are due equally to synonymous and non-synonymous substitutions. There is a bias in nucleotide composition of the sequences in the repeat area and a low content of thymidine was found on the mRNA-like strand in this area in contrast to the rest of the sequence (Arnot *et al.*, 1988). The authors proposed concerted evolution (see 1.3.3.4) processes as the mechanism to maintain homogeneity among the repeats. The biased thymidine content was thought to be generated by the specificity of the heteroduplex repair mechanism and has been observed also in tandemly repeated sequences within the human gene coding for involucrin (Eckert and Green, 1986).

Diversity in this gene has been related to differences in transmission. The CS proteins of parasite isolates originating from areas of high endemicity exhibited more diversity due to point mutations than those coming from low endemicity areas (Udhayakumar *et al.*, 1994; Shi *et al.*, 1992). This amino-acid variation was limited to three clusters in a region known to stimulate proliferation of T cells (Th cells or CTLs). The CS gene diversity seems geographically restricted as revealed in a Kenyan survey in which 12 out of 15 alleles mapping to the C-terminal region were present only in Kenya, while three were universal. This shows the rapid turn-over in the generation of novel sequences. Naturally exposed individuals could recognise five out of eight epitopes tested while, at

the population level, all eight epitopes were recognised. It was not possible to demonstrate if this was due to cross reactivity at the epitope level or subsequent infections with different strains (Udhayakumar *et al.*, 1997).

1.3.3.2. Gene structure of important blood stage antigens

1.3.3.2.1. The gene for the merozoite surface protein 1 (MSP1)

This gene is a large single copy gene in *P. falciparum* that codes for a precursor protein forming the surface coat of the merozoite. Diversity is based on size and sequence polymorphism, and extensive studies resulted in the division of the sequence into 17 blocks (Tanabe *et al.*, 1987). Seven blocks showed high variability and ten had semi-conserved or totally conserved sequences, giving a dimorphic appearance from blocks 5 to 17 (see Fig. 10.) which creates two different allelic forms. A third form was discovered by Peterson *et al.* (1988) but its polymorphism was restricted to block 2.

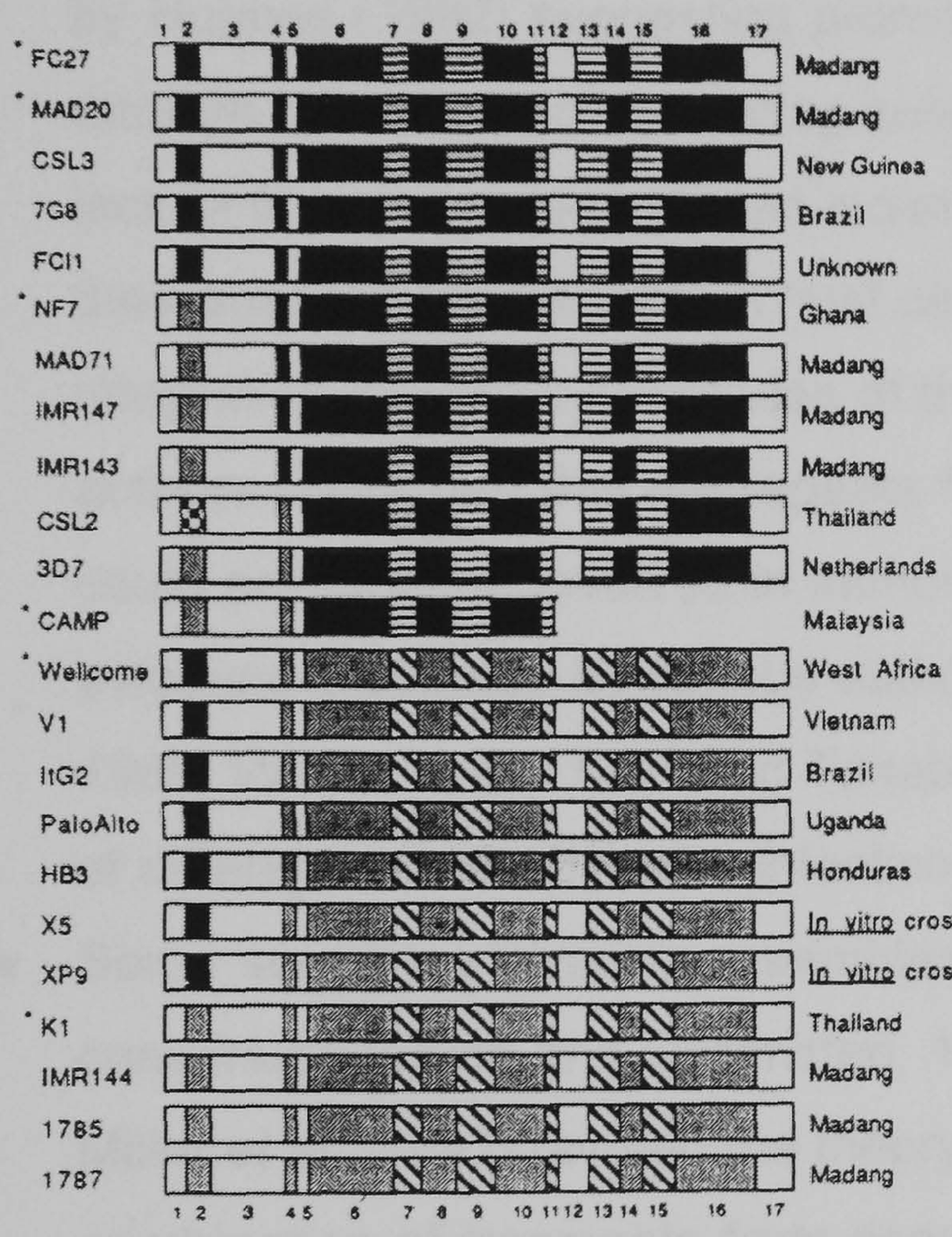


Fig. 10. Structure of MSP1 genes from 24 isolates of *P. falciparum*. The 17 blocks indicated by the numbers at the bottom correspond to regions that differ in homology as indicated by the shading. The darker the shading the more diverse the regions are. Picture from Peterson *et al.*, 1988.

A protective epitope has been found in the well conserved C-terminal cysteine rich region and a small region of variable tri-peptide repeats is found at the N-terminal end, although one group of isolates had no repeats. The main characteristics of this gene can be summarised as follows:

Evidence for intragenic recombination events has been found at four sites (Conway *et al.*, 1991c; Hughes, 1992; Kerr *et al.*, 1994; Kaneko *et al.*, 1996), causing deletion/insertions and di-or trimorphism in some blocks. In all, this polymorphism in different blocks (2 x 3 x 2) gave 12 associative types. In Thailand alone, seven associative types were found (Jongwutiwes *et al.*, 1991).

Presence of defined regions of interspecifically well conserved sequences. These comprise regions coding for functionally constrained protein structures.

- Block 17 is a cysteine rich C-terminal end showing point mutations.
- Evidence for positive selection in the N-terminal region has been presented by Hughes (1992) suggesting promotion of diversity in this repeat region through some form of balancing selection. Certa *et al.* (1987) reported the lack of tripeptide repeats in an isolate from Ghana, refuting the hypotheses of their possible involvement in host cell recognition (Mackay *et al.*, 1985) or integration into the cytoskeleton of the host cell (Cheung *et al.*, 1986). The authors concluded that the repeats found in the other MSP-1 sequences could probably be involved in immune escape events as experimental evidence presented by various authors (Holder *et al.*, 1985; Howard *et al.*, 1986; Weber *et al.*, 1986 and Tanabe *et al.*, 1987) suggested the generation of different repeats by each infection.
- Some sequence diversity resembles patchwork patterns indicative of gene conversion (Erlich and Gyllensten, 1991) or frequent intragenic cross-over. Miller *et al.* (1993) favored the theory of gene conversion involving the combination of fragments from pseudogenes. They rejected the implausibly frequent double intragenic cross-overs required to explain the detected variation and argued that the dimorphism arose from two geographically isolated areas before malaria spread. The diversity of these two alleles was further shaped by gene conversion processes resulting in the exchange of different blocks of sequences, forming a patchwork pattern appearance of similar sequences in the thus created alleles of the two major allelic families.

In a study of 340 wild *P. falciparum* isolates from Southeast Asia and South America, Tanabe (1997) identified > 600 distinct populations (characterised by

associations of different genotypes based on MSP-1 typing) and presented evidence that some MSP-1 genotypes might be favored by natural selection. Kalokovich *et al.* (1996) also proposed gene conversion as the most plausible mechanism for the occurrence of a gene mosaic structure after analysis of the related sequence in *P.vivax*. They also discovered an unusually high and variable number of Q repeats in region 5 of some *P.vivax* isolates, creating a new subtype displaying extensive size polymorphism.

1.3.3.2.2. The merozoite surface protein 2 (MSP2) gene

This gene codes for a smaller protein than MSP1, anchored by a GPI moiety in the merozoite membrane. This gene exhibit significant variability as defined by mAbs profiles, distinguishing two major serotypes. The MSP2 gene has highly conserved terminal regions extending beyond the putative signal and anchor sequences. The central region consists of an extensive variable region, revealing dimorphism with repetitive tetrapeptide sequences varying in number, length and sequence in the ICI/3D7-type. The FC27-type has a more conserved, two repeat structure with a long 32-amino-acid and a shorter amino-acid repeat (including an ICI-like tetrapeptide sequence). This dimorphism extends into the flanking regions and is made up of non-repetitive variable domains (Smythe *et al.,* 1991). Intragenic recombination events between the 2 allelic families have been suspected (Snewin *et al.,* 1991; Marshall *et al.,* 1991). This has generated limited variation but caused a high degree of variability in the repetitive sequence region through numerous deletion and insertion events. All recombination sites lie within the variable region and one has been identified within a stretch of 9 bases that are identical in both alleles (Marshall *et al.,* 1991). This is in contrast with the recombination sites found in the MSP1 gene which are all located within the conserved regions. It has been argued by Smythe *et al.* (1991) that some relationship can be found between repeated sequences and 3' flanking sequences, explaining the origin of some of these sequences. In a more extensive study of sympatric samples, Felger *et al.* (1997) found that all but one of the alleles of the ICI family had novel repeat sequences. The repeat codons showed a strong bias in NNT codon usage which suggests that homogenization mechanisms are at work to maintain DNA sequence homology. The region

seems to be devoid of epitopes. Moreover, no indication of positive selection could be found which is reflected in a 1:1 ratio of synonymous versus non-synonymous substitutions. The major sources of interallelic MSP2 diversity could be related to replication slippage, causing deletions or duplications and mitotic or meiotic unequal exchange leading also to differences in copy number of repeats (Felger *et al.,* 1997). The question as to whether these mutations occur at random (genetic drift) or through frequency-dependent selection has not been answered. On the other hand, monoclonal antibodies raised against the FC27-type have been shown to inhibit parasite growth *in vitro* and map to a 6-mer sequence within the center of the 32-mer repeat (Epping *et al.,* 1988). Another B and T cell epitope has been identified (Rzepczyk *et al.,* 1990 and 1992) in this family and map to the variable central repeat region. Sequence variation was restricted to a few sites, suggestive of possible targets of immune responses. Felger *et al.* (1997) speculated that this repeat region might be under purifying selection pressure, maintaining the two repeat structures. All observed substitutions resulted in non-synonymous changes which suggests the action of positive selection (Felger *et al.,* 1997).

1.3.3.2.3. The var gene family

This gene family (between 50-100 copies corresponding to 6% of the total genome DNA content) codes for highly variable erythrocyte membrane proteins (PfEMP-1). Kyes *et al.* (1997) have demonstrated, using an *in vitro* model, that these undergo antigenic switching at a rate of 2% per generation. The mechanism of differential gene expression is called antigenic variation and plays an important role in the generation of polymorphism in molecules initiating cyto-adherance (Baruch *et al.,* 1995). The genes of variant antigens have been shown to code for proteins mediating adhesion to the vascular endothelium, thereby providing an escape from immune destruction in the spleen (reviewed by Borst *et al.,* 1995).

The different forms of these antigens are encoded by relatively stable allelic variants at several gene loci, each containing two exons. The second exon is highly conserved and codes for a putative intramembrane region. The first exon is highly variable, with short regions of homology, forming two to five Duffy-

binding-like (DBL) domains (Kyes *et al.*, 1997). Short (200bp) sequences from this exon were analysed in a limited sample of geographically diverse isolates (Kenya, Vietnam and Vanuatu), indicating well conserved regions alternating with highly polymorphic blocks, including short deletions. The underlying structure is indicative of a group of antigens exhibiting polymorphism in sequences that could be T or B-cell epitopes (Anders and Smythe, 1989). This underscores the hypothesis that the var gene family must provide a biological advantage which leads to its fixation and wide distribution in the parasite population.

Most var genes are located in the subtelomeric region of each chromosome, closely linked to the rep20 repetitive sequence (Thompson *et al.*, 1997; Rubio *et al.*, 1996) and four of the five so far identified and expressed var genes map within subtelomeric locations. Expression of var genes from a chromosomal domain known for frequent rearrangements has important implications for the mechanism of var gene switching and the generation of novel antigenic and adhesive phenotypes (Fischer *et al.*, 1997). There is good evidence that PfEMP1 is an important immunodominant protective antigen which led Gupta *et al.* (1994) to suggest that this could be the major antigen defining the strain structure in *P. falciparum*.

1.3.3.2.4. The ring infected erythrocyte surface antigen gene (Pf155/RESA)

This gene codes for a protein that is one of the major apical complex (rhoptry, microneme and dense granules) proteins. It is deposited onto the erythrocyte membrane at invasion and appears to be correlated with the acquisition of clinical immunity (reviewed by Perlmann *et al.*, 1989). Approximately 25% of the gene consist of repeated sequences and high antibody levels are induced against conserved epitopes within this antigen. Extensive data on the mapping and immunogenicity of various B and T-cell epitopes are available. The characterisation of the RESA proteins with mAbs revealed a large heterogeneity in profiles. It was concluded that this heterogeneity could reflect differences in the genetic make-up of the host to deal with different epitopes. The question remains if this is due to differences in MHC-II types and/or allotype restriction or due to differences in the host antibody repertoire.

1.3.3.2.5. The serine repeat antigen (SERA) gene

This gene produces large amounts of a protein that is secreted into the lumen of the parasitophorous vacuole of the blood stage parasite. This is another protein of the apical complex protein family. It is a single copy gene, occurring as a series of very different but apparently stable alleles. The central region of the gene consists of short tandemly repeated sequences, which are the major antibody binding sites (Kemp *et al.,* 1987) and show dramatic changes in sequence, length and number of repeats, and in reading frame. Although the repeats can vary markedly among isolates (Cowman *et al.,* 1985), some underlying patterns have been observed, indicating intragenic recombination (Brown *et al.,* 1987) or reading frame changes (Saint *et al.,* 1987). These studies all reported marked differences in antigenicity among the different alleles. The N-terminal 47 kDa domain showed sequence variations among different strains (Morimatsu *et al.,* 1997), the majority caused by deletion/insertion events although amino-acid substitutions by point mutations were detected at 17 positions. The various allelic forms could be grouped into three types which vary by approximately 20% at the sequence level (Nicholls *et al.,* 1988), in marked contrast to the conserved ends of the MSP1 gene.

1.3.3.2.6. A liver stage antigen (LSA-1)

This antigen was detected by immunofluorescence in the parasitophorous vacuole of *P. falciparum* liver-stage parasites (Fidock *et al.,* 1994). It is the only *Plasmodium* antigen expressed solely during this stage and has been found to induce immune responses in the majority of malaria-exposed individuals. The development of a LSA-1 based vaccine constructs is currently been tested (Hollingdale *et al.,* 1998). The terminal regions seem to be very conserved (Fidock *et al.,* 1994; Hughes and Hughes, 1995). The central region is made up by many (up to 86) copies of a 17 amino-acid repeat sequence that is highly conserved in sequence but variable in copy number and contains strong B-cell epitopes (Fidock *et al.,* 1994). Antibodies to the repeat sequence were detected but the region seems to lack T cell epitopes (Nardin and Nussenzweig, 1993). This gene is rich in glutamic acid (E; 20%) and glutamine (Q; 18%) (Zhu and Hollingdale, 1991).

The extensive polymorphism affecting various proteins in *Plasmodium* could often be related to selection pressure and immune evasion. The different antigenic proteins are expressed at different surfaces of different parasite stages and during variable lengths of time. PfEMP1 is located on the surface of the trophozoite infected erythrocyte and exposed for at least 18 hours, much more than MSP (1min) or CS protein (30min). There is good evidence that PfEMP1 is an important immunodominant protective antigen which led Gupta *et al.* (1994) to suggest that this could be the major antigen defining the strain structure in *P. falciparum*. This is a good example of the major influence selective pressure can exert on the population structure of pathogens. Pressures, other than these that are immune-derived, will also contribute to the population structure in malaria. Sudden increases in specific alleles associated with drug-resistant parasites could alter the mating structure as argued by Paul and Day (1998).

It has been suggested that other sequences showing diversity and cross-reaction in repetitive sequences and/or epitopes may also influence host immune responses by overloading the immune system of the host (reviewed by Anders and Smythe, 1989). It is still not clear if individuals could recognise all variant CTL epitopes or if the variability in CTL epitopes abolishes binding to some host HLA determinants.

The structure of *Plasmodium* antigens reflects the imprints of different mechanisms at work in the generation of much needed diversity for evading the host immune responses. The generation and control of repetitive sequences together with deletions or insertions, point mutations and intragenic recombinations are the most obvious ones.

1.3.4. Gene diversity in *Theileria*

Cross-immunity data indicate the existence of *T. parva* stocks with different protection profiles, suggesting antigenic diversity amongst isolates. Several polymorphic antigens have been characterised.

1.3.4.1. The polymorphic immunodominant protein (PIM)

This protein is expressed in the sporozoite and schizont stages (Toye *et al.,* 1991). PIM was shown to be localised on the surface of the schizonts by immunolabelling using mAbs. There is strong evidence that this protein is secreted by the microspheres (Skilton *et al.,* 1998). This is of interest as microsphere associated proteins of related parasites have been shown to be strongly immunogenic (Cesbron-Delauw, 1994; Goodger *et al.,* 1992). Antibodies raised against PIM inhibit sporozoite invasion *in vitro*, but steric hindrance could not be excluded (Toye *et al.,* 1995).

In an attempt to survey the extent of heterogeneity among *T. parva* isolates, mAbs were generated against schizont-infected lymphocytes and were used to separate *T. parva* isolates into four groups (Minami *et al.,* 1983). Shapiro *et al.* (1987) showed that most of the mAbs reacted with the same antigen in Western blot assays using schizont-infected cells. This dominant antigen expressed relative molecular mass (Mr) polymorphism and was further characterised by Toye *et al.,* (1991; 1995 and 1996) as the polymorphic immunodominant protein. PIM was shown to be present in sporozoite and schizont lysates only. There was no indication of glycosylation which could explain the Mr differences. Sequence data from three PIM genes from three different parasite stocks revealed a central variable region flanked by conserved termini. The size polymorphism is thought to be due to variations in the length of the central region (Table 2), containing tandemly repetitive sequences (Toye *et al.,* 1996). The repeat structures could be divided in type I tetrapeptide repeats, type II repeats characterised by three consecutive glutamine (Q) residues in a nine amino-acid repeat and type III repeats containing four consecutive glutamine (Q) residues in a variable repeat sequence (Toye *et al.,* 1995).

T. parva stock	bp	Central variable region repeat sequence
Muguga	603	(GlnProGluPro) x 5
Marikebuni	408	(GlnProGluPro) x13
7014 (Lawr)	762	(GluProGluPro) x 2 (GluProGluPro)

Table 2. Nucleotide length in bp of PCR-amplified product embrasing PIM repeats and amino-acids of PIM repeat sequences of different *T. parva* isolates.

The terminal ends contain point mutations and *T. lawrencei* sequences have more heterogenous ends than cattle sequences. Although this gene is correlated with variability in *T. parva* organisms, Western blot analysis of parent and cloned *T. parva* Marikebuni stock parasites revealed size differences amongst the clones but only one major PIM protein was detected (Toye *et al.,* 1991). It was suggested that certain antigens might be predominant while others might be expressed by a minor sub-population revealed only by cloning. The monoclonal antibodies recognised at least ten different epitopes throughout the PIM molecule, corresponding with regions showing point mutations. They did not recognise any tetrapeptide repeats although the latter reacted strongly with sera from infected cattle (Toye *et al.,* 1996). This agrees with previous observations when antisera from immunised animals were used (Toye *et al.,* 1991). The different mAbs profiles found when testing various isolates indicates the presence of different epitopes on different PIMs (Toye *et al.,* 1996). This antigen has been selected for use in an ELISA-based diagnostic test (Katende *et al.,* 1998). Despite its extensive size and sequence polymorphism, it was by far the most sensitive of the four well characterised *T. parva* antigens tested (p67; p104 and p150) in the development of the ELISA (Katende *et al.,* 1998). This has been ascribed to the alleged presence of immunodominant conserved epitopes across the different stocks (Toye *et al.,* 1996).

Two well conserved introns of 55 and 61 bp were found near the 3' end. Sequence analysis revealed features typical of an intinsic membrane protein that is a potential signal sequence at the N-terminal end, a membrane spanning domain and a short hydrophilic tail (Baylis *et al.,* 1993). The proline rich repeat stretches are thought to adopt a rigid elongated structure and are called P-linkers (Baylis *et al.,* 1993).

1.3.4.2. The p150 protein.

The p150 protein has been identified in the discharges of microspheres soon after sporozoite entry and remains transiently in the cytosol and on the parasite surface (Skilton *et al.,* 1998). The p150 protein is expressed in sporozoites and schizonts. Size polymorphism is absent and far less sequence heterogeneity was detected than in the PIM antigen. The p150 is a microsphere protein of 150 kDa (Skilton *et al.*, 1998), 5 kb of the cDNA comprising the gene has been sequenced and compared between four isolates. The ORF is 4.38 kb long, with strongly conserved sequences and apart from polymorphism in a region near the C-terminal end, is mostly characterised by point mutations from bp 3565 to 4323 and insertions/deletions in a proline rich repeat region starting from bp 3708 to 3849. Five different types (A-E) could be determined among ten different *T. parva* stocks, producing slight size polymorphism (12-96 bp) when the sequences of their repeat region were aligned (Skilton *et al.,* 1998). Type A includes *T. parva* Muguga, Mariakani, Kibarani, Kilifi and Pemba, Type B: *T. lawrencei* 7014-1, Type C: *T. parva* Marikebuni and Uganda, Type D: *T. lawrencei* 7014-2 and Type E: *T. parva* Boleni. There was no indication of a geographical correlation among the different alleles. A serological cross-reaction was apparent between an epitope in the repeat zone of the p150 protein with a similar epitope in the PIM protein (mAb 8).

1.3.4.3. The p104 gene.

The p104 gene is expressed in sporozoites as a microsphere associated protein (Iams *et al.,* 1990). It consists of a 104 kDa molecule coded by a 2772 bp long ORF with 40.8% GC-content and no evidence of introns. A cDNA clone of 4.5 kb, containing p104 was sequenced and the sequences compared between three isolates. The sequence is strongly conserved except for point mutations at 22 places scattered over a 750 bp region up to the C-terminal end. The sequences for this polymorphic region of nine different *T. parva* stocks have been aligned and four types could be distinguished. The groups consisted of the same stocks as found for the p150 polymorphism, except that only one buffalo stock was included (R.Skilton, personal communication). No profiles could be

related with geographic origin. Three antigenic domains were detected by screening of subclones of the gene with antisera and all mapped to the 3' end. Proline rich repetitive sequences were found in two of the antigenic domains and there is good evidence that cleavage occurs producing three immunogenic peptides of different molecular weights (104, 90+14, 85+19 and 35 kDa) as revealed in Western blots (Iams *et al.,* 1990).

1.3.4.4. The major sporozoite antigen (p67)

The sporozoite antigen p67 codes for a protein that forms part of the surface coat of sporozoites. It has been well characterised and shows a remarkable sequence conservation in cattle-derived *T. parva* isolates (Nene *et al.,* 1996). This antigen is not important as a target for the analysis of genetic polymorphism but could separate parasites obtained from buffalo on the basis of an insert which was absent from bovine isolates. Low titres of neutralising antibodies against p67 are found in naturally infected animals. High antibody titres can be induced by using recombinant p67 and these antibodies inhibit sporozoite infectivity *in vitro*. This antigen has been explored as a candidate for a molecular engineered vaccine following the lead of *Plasmodium* research on the CS protein as a vaccine candidate.

1.3.4.5. A major merozoite antigen (p32)

The merozoite antigen p32 was also characterised. The 843bp long p32 gene of *T. parva* Muguga was sequenced at ILRI (R. Skilton, unpublished data; EMBL, accession nr. L47209). This antigen is coded for by one of a group of related genes found in all *Theileria* species and *B.equi*. These proteins have a molecular mass of between 30-34kDa (Kawazu *et al.,* 1992; Shiels *et al.,* 1995; Knowles *et al.,* 1997). They are characterised by their proposed location on the surface of piroplasms, their lack of repeat sequences and their immuno-dominance. Molecular mass differences compared with predicted mass has been observed in these genes in all *Theileria* species and are thought to be caused by differences in glycosylation sites (Kawazu *et al.,* 1992). Restricted fragment lenght polymorphism (RFLP) analysis differentiated clearly between *T.sergenti*, *T.buffeli*, *T. annulata* and *T. parva*, and between *T. annulata* isolates from different countries (Shiels *et al.,* 1995). A region close to the 5' end of the

gene, spanning approximately ten amino-acids, was found to harbor most of the predicted glycosylation sites. This suggests the presence of antigenic epitopes (Shiels *et al.*, 1995) and a diversity generating mechanism, based on selective immune pressure. Sequencing data of a limited number of isolates from different geographical locations for *B.equi* (Knowles *et al.*, 1997) and *T. parva* (own data) revealed a well conserved sequence structure for this gene in marked contrast with the polymorphism found in the p32 sequences of *T. annulata* (Dickson and Shiels, 1993). It was shown by Shiels *et al.* (1995) that Tams1-1 and Tams1-2 sequences coding for the 30 and 32kDa proteins of *T. annulata* respectively are alleles of the same gene, showing molecular mass polymorphism which could be due to differences in glycosylation (Dickson and Shiels, 1993). The same polymorphism was shown in the case of *B.equi* in which different sequences coding for the 30-34kDa proteins were found (Knowles *et al.*, 1997).

These results clearly demonstrate a difference in immunological nature of the same protein in the various *Theileria* species and might reflect an important difference in survival strategy by the different species. The p32 variability found in some *Theileria* species could be correlated with an important role of the piroplasm stage in the pathogenesis of the diseases they cause. It could be that important immune responses against this pathogenic stage would put a gene coding for a major protein under strong selective pressure, resulting in substantial polymorphism which contributes to the evasion of these responses.

The substantial diversity identified through different genome characteristics and revealed using unrelated methods points towards a relatively large degree of polymorphism within the *Theileria* species. This might suggest the importance of immune evasion in the survival and propagation of the parasite, although the natures of the evasion mechanisms are not known. The complexity of the molecular basis of these host-parasite interactions is highlighted by the existence of different immune effector mechanisms and a multitude of epitopes, often within a single gene, most of which seem to be recognised by a single host (Nardin and Nussenzweig, 1993).

Table 3. A summary of the major parallels between the *Plasmodium* and *Theileria* genes.

Gene	Copy number	Organisation	Polymorphism	Mechanisms	Epitopes
Plasmodium falciparum					
CS P. falc	1	CT-CVR	No's of repseq	C-O, PM, I/D	CD8 in CTR; CD4 in repseq
CS P. vivax	1	CT-CVR	48 bp insert / repseq	C-O, PM, I/D	
MSP-1	1	trimorphism		IR/GC, C-O, PM, I/D	CTR, not in repseq
MSP-2a	1	CT-CVR	No's of tetra repseq	IR – I/D	CVR: novel seqs but no epitopes.
MSP-2b	1	CT-CVR	2x Long 32AA reps	UC-O	2 epitopes found in CVR
RESA	1		25% repseq		CD4/8 epitopes
SERA	1	VT-CVR	No's/length and seq of repseq		CD4 in repseq
LSA-1	1	CT-CVR	seq		PM CD4 in repseq; CD8 in CTR
Theileria parva					
P67	1	CT		128 bp insert and seq	PM
P32	1		seq	PM	
P150	1	CT		seq + Nos of repseq	PM
P104	1	CT		seq	PM
PIM	1	CT-CVR	seq + No's of repseq	C-O, PM, I/D	CD4

CT: conserved terminal ends; CVR: central variable region
C-O: cross-over; PM: point mutations; I/D: insertion/deletions; IR: intragenic recombination; GC: gene conversion; UC-O: unequal C-O.
CTR: C-terminal region.

1.4. Parasite populations

1.4.1. Parasite population structure

The parasite population structure is derived from the genetic make up of the individual members of this population in the field and may result in two extreme forms called clonal and panmictic population structures. The frequency of sexual reproduction in the parasite's life cycle is the main determinant of the population structure. In parasitic protozoa such as *Plasmodium* and pathogenic *Theileria* species, sexual reproduction is an essential part of their life cycle. But sexual reproduction frequency is not the only determinant of the population structure as the number of parasite genotypes present in the host will limit the possibilities of mixing during sexual reproduction in the vector. The transmission intensity, determining the chances of two different genotypes meeting in the same host, will be a major factor. Epidemiological and immunological factors will also influence the transmission intensity (reviewed by Hastings and Wedgwood-Oppenheim, 1997). The epidemiology will affect transmission because of various barriers to the free flow of genes between sympatric (within same location) parasite populations. The most obvious are geographical isolation by physical barriers or genetic isolation by biological barriers, but low infection rates, differing host preferences or an epidemic population structure would all contribute to reduced transmission. Appraisal of a situation in which a biological or immunological barrier influences the population structure is difficult. The immunological barrier is mediated by acquired immune responses in the host against a single immunodominant locus (Gupta and Day, 1994; Gupta *et al.*, 1994) or against several immune loci (Gupta *et al.*, 1996).

In most protozoa belonging to the class *Sporozoea*, erythrocyte stages, even those originating from a cloned infection (Walliker *et al.*, 1973), will differentiate into micro- and macro-gametes. After syngamy in the vector, these will form a diploid zygote and give rise to thousands of haploid sporozoites following multiple divisions, including meiosis. In the case of *T. parva* the syngamy between two gametes occurs in the gut of the tick after feeding on a single host.

Cross-fertilization between different genotypes has been shown under laboratory conditions (Morzaria *et al.,* 1992b). Gametes of the same genotype (selfing) or from different genotypes (outbreeding) can form a zygote. When selfing occurs, the progeny will have the same genotype as the parents, apart from a few mutations spread over its genome. Two different genotypes need to be present in the same host for outbreeding to occur, probably in semi-equivalent proportions. This has been confirmed for *P. falciparum* by Ranford-Cartwright *et al.* (1993). They showed that when two clones of equal frequency mate randomly in the vector, half of the zygotes are inbred and half are crosses. Differences in the temporal occurrence of infecting populations within the host will affect the population genetics of these parasites.

Co-infections

The prevailing epidemiological situation is the most important factor determining the transmission intensity and hence the occurrence of co-infections (two different genotypes occurring simultaneously in one host) by different strains. This has a major impact on the diversity found in field populations and has been shown in studies of population genetics of different protozoa (Babiker *et al.,* 1994; Paul *et al.,* 1995). Shi *et al.* (1992) found that *Plasmodium* isolates from highly endemic areas were more diverse than those from areas showing low endemicity. The determination of the mean and the distribution of the number of genotypes per host is an important parameter in analysing the population structure in the field (Paul and Day, 1998).

Super-infections

The role of super-infections (two different genotypes infecting a host sequentially) in diversity generation is principally determined by the immune responses of the host towards the specific parasite and only secondly by the transmission probabilities (Gupta *et al.,* 1994). The extent of cross-protection will influence the entry and establishment of new parasite populations in the infected and recovered host, and plays a more important role in diversity generation than transmission intensity. In its extreme form it will lead to a clonal population structure of the parasite population.

Data from field surveys indicate that most multiple clone infections with *P. falciparum* are derived from multiple infected mosquitos and not from super-infections (Conway *et al.,* 1991b). Under low transmission intensity, the role of super-infections will be the only relevant contribution towards genotype diversity as co-infection possibilities will be very low.

A special form of clonal structure can be found in the case of an epidemic, caused by a new successful immuno-variant. This variant can become the dominant genotype temporally due to the presence of a high percentage of susceptible animals at the time of its appearance. This will cause the emergence of a population structure resembling clonal propagation also known as an **epidemic population structure** (Maynard Smith *et al.,* 1993).

In the case of the lower eukaryotes such as *Plasmodium*, *Babesia* and *Theileria*, complete panmixia will not occur as a considerable percentage of mating will occur between identical genotypes. Field data from *Plasmodium* infections reveal important homogeneity in kinetes indicating a high rate of selfing (30-90%), especially in areas experiencing low transmission intensities (Paul *et al.,* 1995; Hill *et al.,* 1995). The frequency of single clone infections resulting from selfing was found to range between 20 and 70% for *P. falciparum* and around 80% for *P. vivax*. Data from population genetics studies in endemic areas indicate that 60% of all *P. falciparum* infections are multiple, consisting on average of two to three different genotypes (reviewed by Babiker and Walliker, 1997).

Many confounding mechanisms may be at work biasing mating towards self fertilisation. Strain-specific immunity, sequestration mechanisms and various interactions between parasite populations within mixed infections might all limit outcrossing possibilities. Parasite interactions have been studied by Taylor and Read (1998) and Taylor *et al.* (1997a and 1997b) using *P. chabaudi* as a model. Total transmission from mixed infections was found to be higher than from single-clone infections (Taylor *et al.,* 1997a) and less abundant clones often achieved a higher transmission success (Taylor *et al.,* 1997b). This could be related to an asexual parasitaemia on the day of the feed. The authors favored

the differential immune hypothesis to explain density differences observed between clones, concluding that strain-specific immunity might be the most important factor (Taylor and Read, 1998). Although no similar studies are available for *Theileria* infections in ticks, it is also possible that a similar strain-specific immunity occurs.

In population genetics, the recombination rate between two different populations is defined by the expected frequency of re-arrangements between two loci and the outbreeding rate. Non-random association of alleles is measured by the degree of linkage between the various loci. Linkage disequilibrium (LD) is strongly indicative of deviation from random mating, but is not necessarily evidence for non-random mating. In practice, it has been difficult to interpret linkage analysis as other mechanisms can influence the randomness of allelic associations. Linkage disequilibrium might arise when samples are derived from subpopulations with different allelic frequencies and not recognised as such. Limited gene flow between geographically isolated populations can give LD due to selection or drift. Infrequent mixing of genotypes in parasite populations, mainly due to transmission constraints, can also give LD. Alternative gametocyte sex ratio's could be used to explore population structures as these showed a highly female bias when studied in a high inbreeding malaria population. This was also predicted by the sex allocation theory under inbreeding conditions (Read *et al.,* 1992 and 1995).

Many data are available from epidemiological surveys on allelic prevalences in parasite populations causing various diseases, particularly malaria, that have been reviewed by Babiker and Walliker (1997) and Tibayrenc (1998). These data, obtained using different markers and techniques, reveal the existence of complex and highly diverse *P. falciparum* populations in the field. This has been confirmed by molecular characterisation of infections in mosquito's, showing recombination between parental genotypes (Babiker *et al.,* 1994). But under certain epidemiological situations, there are strong indications that clonality might prevail (Tibayrenc *et al.,* 1990 and 1991; Paul *et al.,* 1995). The controversy spawned by these different findings (Tibayrenc and Ayala, 1991; Walliker, 1991) is indicative of the recent and fast developments in the field of population

genetics of pathogenic microorganisms through the availability of powerful tools and methods of molecular biology and statistics.

The presence of mixed parasite populations in field isolates has been reported for *T. parva* (Toye *et al.*, 1991) and *T. annulata* (Shiels *et al.*, 1986). The first extensive survey on *T. parva* diversity came from a Zimbabwe study (Bishop *et al.*, 1994) on 40 cell culture isolates, using a panel of mAbs and three genomic probes (Telomere, Tpr1 and ribosomal probes) to generate RFLP profiles. The telomere probe showed the highest discriminatory power, with identical Tpr1 profiles for all bovine isolates from areas where buffalo were absent. This is in contrast to most Kenyan isolates which showed markedly different Tpr1 profiles (Conrad *et al.*, 1987; Bishop *et al.*, 1993b). The majority of buffalo-derived stocks showed different profiles on Tpr1. Based on the similarity among Tpr1 profiles and mAbs, the authors concluded that the bovine *T. parva* isolates showed good homogeneity, in contrast to Kenyan data and buffalo-derived *T. parva* from Zimbabwe. These results indicated that selection for a particular Tpr1 genotype may be occurring in cattle. Results obtained with a species specific probe also showed that *T.taurotragi* was widely present in the field in Zimbabwe and confirmed the presence of the Tpr multicopy gene family (Baylis *et al.*, 1991) in *Theileria* species, other than *T. parva*.

Ben Miled *et al.* (1994) reported on the characterisation of *T. annulata* field isolates from Tunisia using phenotypic (mAbs against macroshizonts and GPI isoenzymes) and genotypic (two untyped probes) markers. The mAbs differentiated between two phenotypes and the results strongly suggested the presence of mixed parasite populations in field-derived macroschizont-infected cell lines. The results with the four different markers showed that none of the 53 isolates had the same profile, even from animals on the same farm. Four different genotypes were found in one isolate. These characterisations were done on cell culture isolates and selection by the *in vitro* isolation technique could have reduced the initial diversity. Data were presented also which are suggestive of selection in populations between the schizont and piroplasm stages. This might be correlated with selection acting in cell culture systems or

with differences in merogony *in vitro* between parasite clones as demonstrated by Shiels *et al.* (1992). Loss of parasite subpopulations within a field isolate have been reported during cloning (Le Bras *et al.*, 1983; Conrad *et al.*, 1987 and 1989; Viriyakosol *et al.*, 1994) or through treatment of *in vitro* cultures (Brown *et al.*, 1989). A subsequent survey in Tunisia, characterising *T. annulata* isolates with four different genomic probes (Tams1, rhoptry, RNA and SPAG-1), showed extensive polymorphism with no correlation between genotype and geographical region (Shiels *et al.*, 1995).

Evidence of complexity was also found in *T. sergenti* in which most stocks were shown to consist of two populations when characterised using the p32 antigen (Kubota *et al.*, 1996). When infection from ticks and blood were compared over time in carrier animal population, the ratios of the two p32 alleles changed.

There are sufficient data indicating the complexity of *Theileria* population structures in the field and this has been accepted throughout the ECF distribution as the paradigm. Evidence for a multi-component strain structure comes from various studies. Immunologically different populations, which might be selected by cloning, were found in samples from carrier buffalo (Conrad *et al.*, 1989). *Theileria parva* Mariakani and Marikebuni have been shown to be composed of at least four different clones (Conrad *et al.*, 1989; Toye *et al.*, 1991). There is evidence that epidemiological differences might influence this diversity (Bishop *et al.*, 1994) as has been shown already in *P. falciparum* field surveys (Paul *et al.*, 1995; Hill *et al.*, 1995; Shi *et al.*, 1992). It has been common practice to extrapolate findings from one epidemiological situation and apply these to the total range of the pathogen. There is a real danger in generalising data defining the composition of parasite populations in one epidemiological area to the whole area, especially in the case of the application of extensive immunization with composite vaccines, using stocks from very different epidemiological backgrounds.

1.4.2. Population genetics and dynamics of parasitic protozoa

Population genetic studies provide data on allele frequencies of various loci. These data can be analysed further to determine general gene diversity within populations. The analysis of allele frequencies will give insights into the population structure supporting this frequency distribution and possible concurrent distorting mechanisms. Allelic frequencies can change through random genetic drift, mutation and selection pressures, migration or vaccination. Random drift will favor certain genotypes over others in small populations as true randomisation of mating is restricted in limited parasite populations. Migration will contribute to an accelerated gene flow between various populations whereas vaccination imposes increased selection pressure to evade the created immune state. In the case of immunisation using live parasites, additional disturbances might be created through the introduction of new genotypes into the resident host population and through recombination with wild-type parasites. This is especially so when using cocktail-based vaccines. On the other hand, restrictions of gene flow will occur through geographical or biological barriers. As a consequence, population genetic studies should incorporate sufficient sympatry in sample collection in relation to existing geographical barriers and good knowledge of parasite and vector species present. Population dynamics studies provide data on the allelic frequency variation in time through selection or chance mechanisms, thereby bringing an important insight in past and future changes.

1.4.2.1. Population mating structure

The population mating structure can be derived from an analysis of allele frequencies in populations. Random mating resulting in constant reshuffling of the gene pool (panmixia) is thereby taken as the null hypothesis. Departure from the null hypothesis is suspected when multi-locus genotypes appear to be related among individuals in a population. This is known as linkage disequilibrium (LD). The power of tests to detect significant diversity depends on the markers used. The degree of polymorphism of markers should reflect the level of resolution required. This is determined by both the inherent variability of

a gene, called the molecular clock of a gene (Zuckerkandl and Pauling, 1965; Kimura, 1987), and the linkage between different genes. The molecular clock is a measure of the speed of variability (evolution) displayed by a gene. The factors influencing the speed of the clock of a gene depend on its position within the genome, coding capacity, functional constraints and antigenic value. It has not been easy to identify and quantify all these influences, and several tests are described which emphasise different possibilities (reviewed by Tibayrenc, 1995 and 1996). If the extent of linkage (disequilibrium) of genotypes varies between different populations, this could indicate different mating systems or parasite population sizes in these populations. If different loci appear differently related between individuals of the same population, this may be suggestive of active selection mechanisms, provided other barriers have been excluded.

1.4.2.2. Population dynamics

An understanding of the dynamics of the various mechanisms influencing allelic frequencies is necessary for a sensible and operational interpretation and use of the data. The importance of most mechanisms responsible for variation is dependent on the parasite's effective population size (Ne) and the generation time (T). Ne depends on the male/female ratio, the portion of reproductively active individuals in the population and the mating frequency. It follows that in the case of *Theileria* parasites, Ne will be close to the real population size N and is calculated as the harmonic mean of the different generation sizes (Li, 1997). This implies that Ne will be closer to the smallest value of N than to the largest one. T is characterised by the time spent in the host prior to its transmission to the next host. For *P. falciparum*, this has been estimated at a minimum of four weeks, but eight weeks would be a more realistic figure (Hastings and Wedgwood-Oppenheim, 1996). It can be seen that this parameter will depend on the transmission intensity these parasite populations are subjected to. Changes due to random mutations or genetic drift become important when Ne is small and T is short, contrasting with the lack of influence the population size has on selection. Here the fitness advantage and the initial frequency are important. The time to fixation depends on the frequency of the allele, the Ne and the fitness advantage. Gene substitutions are dependent on their fixation

probability and fixation time, defining the rate of substitution. This rate is independent of population size in the case of neutral mutation, but depends on Ne and the selective advantage in the case of selection.

Selection can be purifying (selection against deleterious mutations), balancing (same fitness) or neutral (no fitness advantage). In the last case, chance dependent mechanisms such as random drift become important in allele frequency changes. Balancing selection will be correlated with important immunogenic loci as rare genotypes will become more successful with increasing numbers of susceptible hosts. This leads to long term maintenance of such alleles and is an important mechanism in maintaining polymorphism. At the population level, Kimura (1976) estimated that most neutral mutants will be lost within ten generations and only a minority will become fixed over a long period (100.000 generations). In contrast, if a mutation is advantageous, its ultimate fixation probability is estimated at twice its selective advantage (Haldane, 1927), meaning that a mutation with 1% selective advantage will be lost in 98% of the cases.

The second important factor in population dynamics is the rate of recombination in the population under study. Recombination is used here to mean the frequency of reassortments between two loci. The rate (r) is determined by the physical linkage between two loci, multiplied with the outcrossing rate (o) or true recombination rate. Physical linkage relates to the proximity of two loci on the same chromosome. The recombination rate between two unlinked loci in the case of absence of inbreeding is 50% per generation, giving r = 0.5 as the maximum possible recombination rate. If only 30% of the reproduction involves different genotypes, then the true recombination rate is 0.5 x 0.3 = 0.15 per generation. This is a very important concept in population genetics of protozoans as the true recombination rate will influence the lifespan of a particular genotype (strain). Modeling by Hastings and Wedgwood-Oppenheim (1996) suggested a base line of $r<0.001$ for the recombination rate under which clonality will prevail. They estimated that in the case of *P. falciparum*, strains will be stable for at least 20 years when r = 0.01. If an epidemic situation prevails, the occurrence and

extent of epidemics is another factor determining clonality. The same authors proposed a quantitative model which defines the impact of epidemics (sp) as the probability (s) of an epidemic occurring in a generation multiplied by the resulting proportion (p) of the population involved. Bigger 'sp' values will counter the effects of recombination and a strain structure can arise even when recombination rates are above the threshold. If an epidemic occurs every 100 generations (s=0.01) and affects 1% of the population (p= 0.01), the 'sp' value will be 10^{-4}.

1.4.2.3. Population genetics and the molecular strain concept

The approach to delineation of parasite populations for phylogenetic studies is different than for epidemiological studies. The latter involves the search for markers with linkage to the phenotype. The basic strain concept is derived from the fact that frequent genetic exchange will oppose the emergence of discrete lineages or strains in a population. Parasite populations will not become structured above a certain frequency of genetic exchange that will prevent or destroy the formation of discrete lineages.

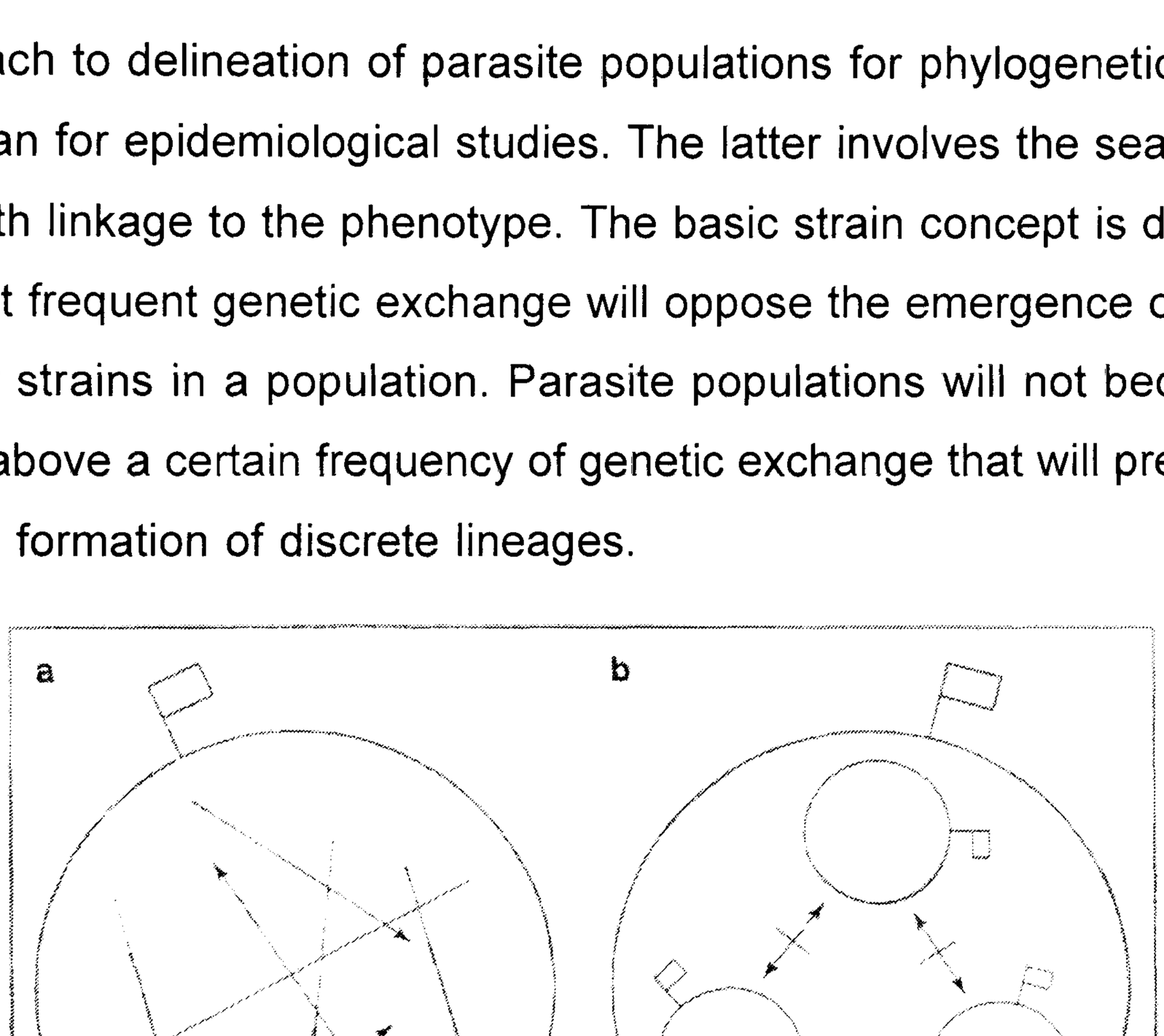


Fig. 11 Diagrammatic representation of nonstructured (a) versus structured (b) models of pathogen populations[3]. In the nonstructured model, genetic exchange (symbolized by arrows) is frequent enough to prevent the maintenance of discrete genetic lines. The operational unit of research is the whole species only. In contrast, in the structured model, the species is subdivided into discrete genetic entities between which gene flow (symbolized by double arrows) is inhibited. In the two models, the species are supposed to be monophyletic and, therefore, correspond to 'discrete typing units' (DTUs)[6,7] that can be specifically identified by appropriate genetic markers or 'tags' (symbolized by small flags). In the structured model, the species is further subdivided into lesser DTUs, each characterized by specific tags. These lesser DTUs can be taken as units of research as well as the whole species.

Figure 11. Model of a structured and nonstructured parasite population, from Tibayrenc, 1998.

Characterising lineages in parasite populations depends on the availability of (i) well defined markers to delimit species boundaries and (ii) markers of adequate resolution required to characterise the relevant lineages (Tibayrenc, 1998). The first step consists in defining a set of markers common to all genotypes present in a species.

These markers will possess essentially low resolution characteristics and relate to DNA loci displaying a slow molecular clock. If mechanisms are at work that restrict frequent gene flow between genotypes, the species will be subdivided into discrete entities, each characterised by a different set of high resolution markers (see Figure 11). This concept defines strains as discrete lineages (different groups consisting of genotypes identical for a defined set of markers) of a species which retain their specific identity long enough to have an operational function in epidemiological or clinical terms. Genotyping studies can be very misleading if nothing is known about the stability and resolution of the markers used. Insight into the stability of genotypes can be derived from the population mating structure, if known, whereas the resolution level of markers could be inferred from evolutionary genetics if sufficient data are available.

An important factor in the 'epidemiological' strain concept is the extent of genomic polymorphism. Strains are defined by multiple loci which have not been quantified for most pathogens. These loci might include genes controlling virulence and clinical characteristics. Recombination rates will influence the linkage between the various important loci. The rate of recombination necessary to prevent this linkage will predict the presence of differences in virulence and transmissibility within a strain, and contribute to the understanding of differing clinical syndromes. Strains could be characterised by different genotypes but have identical virulence properties. If the recombination rate falls under $r<10^{-4}$, linkage between all loci will be expected as determined by Hastings and Wedgwood-Oppenheim (1996). The number of loci involved in the genesis of strains and the outcrossing rate will determine the ultimate differentiation of the population into strains.

An important consequence of these findings is the design of population genetic analysis tests that are capable of identifying immunologically important loci. Hughes and Hughes (1995) presented a test based on the determination of the rate of molecular evolution, better known as the molecular clock of a gene, to track these loci. Gupta *et al.* (1996) suggested the use of linkage disequilibrium between putative immune loci in the search for important loci and Hastings and Wedgwood-Oppenheim (1996) proposed the differences in genetic variation within hosts and around putative loci in the parasite as a way to identify such loci.

The possibilities of testing hypotheses regarding the interactions of genetically different parasite populations and their transmission patterns have been shown in various analysis of field survey data using genotyping of *Plasmodium* parasites (Conway *et al.,* 1991b and 1992; Conway and Bride, 1991a; Ranford-Cartwright *et al.,* 1993; Babiker *et al.,* 1994; Ferreira *et al.,* 1998; Kyes *et al.,* 1997), *Borrelia* (Qiu *et al.,* 1997), *Trypanosoma cruzi* (reviewed by Tibayrenc, 1998). Ferreira *et al.* (1998) reviewed the current state of MSP-1 knowledge and reported that nearly all (23/24) possible genotypes have been found in a mesoendemic situation in Vietnam, whereas only half of them were encountered in hypo-or holo-endemic situations in Brazil or Tanzania respectively. This has been ascribed to the more selective and sequential use of antimalarials in Vietnam in the context of multi-drug resistance, leading to an increase in genetic diversity in the parasite populations (Ferreira *et al.,* 1998). Random associations between variable blocks would be expected to occur in the absence of selective pressures and was found for the Brazilian and Tanzanian samples. Results from genotype analysis of these data also challenged the hypothesis of independent transmission of various strains (genotypes) as indications for associations between genotypes was found in Tanzania. Qiu *et al.* (1997) reported on the changing genotype frequencies in *Borrelia* populations in Northern America using the highly variable ospA locus and found indications of balancing selection in maintaining this diversity. A simultaneous temporal variation in genotypes over the whole area suggests that selection mechanisms against the background of moderate gene flow rates are important in these

populations (Qiu *et al.*, 1997). The situation is different in *Trypanosoma cruzi* in which natural populations show considerable genetic polymorphism as a result of long-term clonal evolution with rare events of hybridization between different clones contributing to the generation of families of genetically related clones (clonets). These can be characterised by different sets of high resolution markers (discrete typing units or DTUs), showing longterm stability (Tibayrenc, 1998).

This underlines the important contributions to many research fields that can be expected from population genetic studies and from the powerful molecular-based research tools that have been developed. It is also a good illustration of the benefits of a multi-disciplinary approach and the integration of these data into the research on pathogens.

1.5. Genetics of Theileria parva: genome structure and map

1.5.1. Current characterisation methods in *Theileria parva* research

1.5.1.1. Biological markers based on phenotypes

- Different clinical and parasitological characteristics have been attributed to different *T. parva* parasites. Clinical parameters have been used to determine differences based on the parasitosis, parasitaemia, haematocrit and pathological lesions. Care is necessary in using such characteristics as the initial sporozoite dose can seriously influence these parameters. Two distinct groups could be defined, one with consistently low parasitosis (previously known as the *T. parva* bovis type) and one with very high parasitosis but generally no serious impact on the haematocrit (Uilenberg, 1981). It is not always easy to distinguish between these two groups as very acute *T. parva* infections can kill animals before a parasitaemia has had a chance to develop. A geographical correlation seems apparent and could be the result of parasite evolution under different epidemiological conditions. It is not known if this difference is due to a faster multiplication of the shizont stage or due to the selection of a different genotype changing the interactions between parasite and host. Nothing is known of the stability of the

characteristics resulting in this different phenotype. Although the outcome of the infection is different, distinct clinical syndromes, attributable to different stocks, could not be described because of the variability in lesions. The dose is certainly one of the factors contributing to this variability.

- Cross-immunity data distinguishes immunologically different stocks but is impractical for use as a screening tool for characterisation.

1.5.1.2. Genetic markers based on genotypes

- Zymograms detect strain differences in *T. annulata* (Melrose *et al.,* 1980 and 1984) but do not differentiate among *T. parva* stocks (Musisi *et al.,* 1981; Allsopp *et al.,* 1985). Irvin (1987) associated the lack of diversity in enzymes to the rather recent history of *T. parva* in cattle in contrast to *T. annulata*. Zymograms are also difficult to obtain as *T. parva* piroplasms are not very metabolically active.
- Characterisation of *T. parva* isolates became possible once serological techniques based on mAbs against polymorphic antigens were developed. Shapiro *et al.* (1987) characterised the protein PIM recognised by anti-schizont mAbs and found differences in molecular weight (Mr) and size between different stocks, but no correlation between Mr and cross immunity. Toye *et al.* (1991) showed that the PIM is present in both sporozoite and schizont stages in all *T. parva* and *T. lawrencei* stocks and was the only antigen reacting with the mAbs. Analysis by two-dimentional polyacrylamide gel electrophoresis and immunoblotting, used by Sugimoto *et al.* (1992), confirmed these results. The most extensive results came from a study by Kishima *et al.* (1995). The PIM protein showed size differences (between 83-100kDa) when immune sera from animals immunised with different stocks were examined in two-dimensional Western blotting (Table 4). Strain differences could be detected using several mAbs raised against different PIM epitopes.

	Mug	Mariak	Mbuni	Ug	Bol	Lawr
PIMsize (kDa)	86	88/83/75/69	83/75/69	83	83/75	100
mAb5	*	not done	nd	nd	*	*
mAb7	*	nd	nd	nd	-	*
mAb21	-	nd	nd	nd	*	*

Table 4. Sizes (in kDa) of PIM proteins and mAbs profiles of different *T. parva* stocks. Mariak = Mariakani; Mbuni = Marikebuni; Ug = Uganda; Bol = Boleni; Lawr = Lawrencei. Nd=not done; *=reaction; -=no reaction.

This study also revealed the individual differences in immune responses between animals to the same immunising stocks showing additional or differently sized immunodominant proteins (Table 5).

Stock	Muguga	Mkani	Mbuni	Ug	Bol	Lawr
Sizes		150		150		170
80-100 range: all	*	*	*	*	*	*
		69-75	69-75		75	
		48-55	40-50	48-55	56-60	69-75
	25-32	–	20-30		38	–

Table 5. Sizes (in kDa) of immunodominant proteins in schizont lysates of different *T. parva* stocks. Mariak = Mariakani; Mbuni = Marikebuni; Ug = Uganda; Bol = Boleni; Lawr = Lawrencei.

- Molecular biological techniques revolutionised the possibilities for characterisation and the development of different tools for use in *T. parva* has been discussed under sections 1.3.2. and 1.3.4. The development of a low resolution *T. parva* genome map enabled the determination of the position of most markers on the individual chromosomes as illustrated in Figure 12 (Nene *et al.*, 1998).

T. parva genome location of probes and markers

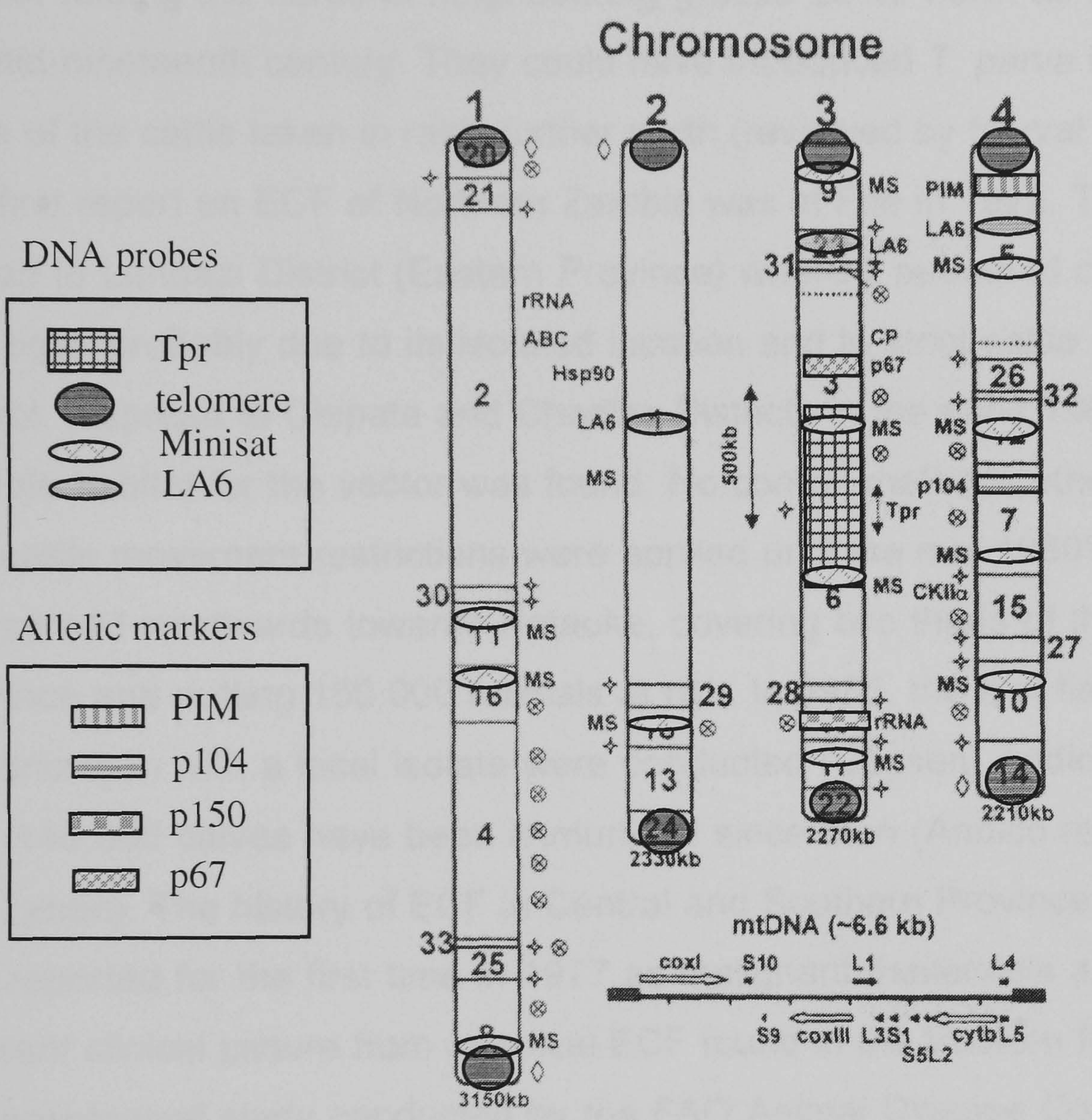


Fig. 12. Genome map with position of discussed markers. Map picture from Nene *et al.*, 1998

1.6. History of East Coast Fever in Zambia

The introduction of ECF into Zambia is thought to have been related to the migrations of the Ngoni people who populated the region of northern Malawi, southern Tanzania and eastern Zambia. They were a bellicose, pastoralist people, raiding the herds of neighbouring groups as far north as Lake Victoria in the mid-nineteenth century. They could have introduced *T. parva* into Zambia in some of the cattle taken in raids further north (reviewed by Norval *et al.,* 1992). The first report on ECF of Northern Zambia was at Fife in 1922. The disease spread to Lundazi District (Eastern Province) where it remained confined for a long time, probably due to its isolated location and to strict cattle movement control. It spread to Chipata and Chadiza Districts in the early 1960's where suitable habitat for the vector was found. No control methods other than dipping and cattle movement restrictions were applied until the mid 1980's. By then it had spread westwards towards Petauke, covering two thirds of the Eastern Province and putting 150,000 animals at risk. In 1985, the first field trials using immunization with a local isolate were conducted (Geysen, Andico project) and over 140,000 calves have been immunised since then (Andico reports, Berkvens and Lynen). The history of ECF in Central and Southern Province is less clear. It was reported for the first time in 1977 as malignant theileriosis and had a different clinical picture from classical ECF found in the Eastern Province. An epidemiological study conducted by the FAO Animal Disease Control Project reported a low incidence rate and marked variation in intensity of clinical disease (Moorhouse *et al.,* 1985). Mortalities occurred in animals of all ages and could reach more than 30% in newly infected areas. The authors concluded that ECF was epidemic throughout the infected area. Immunisation trials were initiated with an exotic trivalent cocktail (Muguga cocktail) of stocks and involved 1,000 animals near Choma and 6,000 animals around Monze.

2. Objectives of the study and materials and methods

2.1. Objectives of the study

1. **To study the genome stability of selected *T. parva* stocks and clones over time**. In order to evaluate the usefulness of the DNA-based markers, genome stability was studied over a two year period by examination of sequential six monthly samples from continuously growing cell cultures of 13 stocks and their 'clones'. These were characterised at different regions and loci by using Southern blot hybridisation with four radio-labeled probes (Telomere, Tpr1, LA6 and Minisatellite, described under 1.3.2 and 2.2.3.4). The probes were choosen for their reported capacity of revealing polymorphism among different isolates (Conrad *et al.*, 1989a; Bishop *et al.*, 1993b and 1994).

2. **To study the potential of molecular tools based on polymorphic antigens in characterising field populations of *T. parva***. In order to characterise parasites in the field, methods using small amounts of easily transported samples are crucial. A major drawback to current *T. parva* parasite characterisation methods using DNA probes is their restricted application because of the requirement for large amounts of parasite DNA. Although *in vitro* cell culture methods are available to generate large numbers of parasites as a source of DNA, their use can present the possibility of clonal selection of parasites (Sutherland *et al.*, 1996; Viriyakosol *et al.*, 1994). Very sensitive diagnostic assays for the characterisation of parasites became available through the development of the PCR technology (Saiki *et al.*, 1988). PCR methods were described to detect *Theileria* parasites in both the bovine host (Bishop *et al.*, 1992; Tanaka *et al.*, 1993) and the tick vector (Chen *et al.*, 1991; d'Oliveira *et al.*, 1995).
   With the development of PCR methods, parasite characterisation using small amounts of sample material is feasible and its application to the field will contribute substantially to the understanding of the epidemiology of theileriosis, caused by *T. parva*.

Characterisation of parasites using amplified polymorphic loci from antigenes is increasingly being used to analyse diversity in the field. Molecular based assays were developed using different methods to survey sequence polymorphism. One or a few partial or full sequences were available for most of the *T. parva* loci studied. These were all generated at ILRI, Nairobi as part of its research programme to define the molecular structure of *T. parva* and develop a subunit vaccine against the parasite. Most of the sequences code for immunological important genes, the rest are sequences generated as linking clones (L1-L8) during the construction of a *T. parva* genome map (Morzaria and Young, 1992a). Initially, PCR protocols developed at ILRI were used to amplify DNA which could be studied for polymorphism using normal gel electrophoresis, heteroduplex (Delwart *et al.*, 1995) and single strand conformation polymorphism (SSCP) analysis methods (Orita *et al.*, 1989). When sequence data were available, appropriate endonuclease enzymes for use in RFLP-PCR analysis were identified by means of a computer programme (GeneJockeyII, Biosoft, Cambridge). When more sequences were available, primers were developed or improved by using a computer programme (PC-Rare, Eurogentec). This led to the development of appropriate assays for a study of the diversity at three polymorphic antigenic loci. Stocks were characterised using multilocus fragment length polymorphism (RFLP)-PCR analysis on these loci (PIM, p104 and p150, described under 1.3.4 and 2.3.2.2.1). A single strand conformation polymorphism (SSCP) analysis technique was choosen to characterise the p32 locus due to paucity of sequence data. Additional sequence determination was done to confirm the results from p32 analysis, to determine the identity in more detail of recent Zambian isolates or to study the underlying diversity of the PIM locus.

Locus	coding	basis	length	alleles	species spec	expression stage
PIM	Ag	size/seq	1000bp	13	+	sp-sch
P104	Ag	seq	1000bp	4	+	sp
P150	Ag	seq	1100bp	4	+	sp-sch
Tpr	?	size/seq	var	6	-	piro
p32	Ag	seq/size?	850bp	2	+	piro
p67	Ag	size	380/460bp	2	+	sp
L1-2	?	seq	250bp	3	+	n/a
L3-4	?	seq	250bp	3	+	n/a
L5-6	?	seq	250bp	3	+	n/a
L7-8	?	seq	250bp	3	+	n/a
ABC cassette	HK	seq	250bp	?	?	?
Telomeric ORF	?	?	?	?	?	?
Chromo1 ORF	?	?	800bp	?	?	?
Schizont expr gene	?	?	700bp	?	?	?

Table 6. Summary of *T. parva* genes and loci used in this study. Ag: antigen; seq: sequence polymorphism; sp: sporozoite stage; sch: schizont stage; piro: piroplasm stage; var: variable; HK: housekeeping gene.

Cloning and sequencing of the p32 locus of two Zambian stocks were performed at ILRI in order to evaluate the SSCP results. The PIM locus showed remarkable size polymorphism, which was studied further by cloning and sequencing of several Zambian stocks. Cloning was done at ITM (except for two recent isolates) and sequences were obtained through the molecular sequencing unit of Eurogentec (Belgium). In all, PCR products of six stocks were cloned and 11 PIM sequences were obtained.
In order to differentiate the complex Theileria field situation a species specific assay (18S assay) based on an alignement of several published theilerial sequences of the SSU rRNA gene retrieved from the Genbank was developed.

A DNA extraction method for use on blood impregnated filter paper was adapted and evaluated at ITM (de Almeida *et al.*, 1997). As the majority of animals in the field are carriers of *T. parva* with very low parasitaemia, the sensitivity of the PCR assays needed to be increased. This was achieved by developing new primers to be used in a semi-nested PCR approach, consisting in two rounds of amplification with different primers, using part of the DNA product generated during the first PCR amplification. The primers

were designed in such a way that the second round used one primer of the first pair and a new primer internally positioned to the other primer of the first pair. The sensitivity and applicability was confirmed in samples from experimental infections and from a longitudinal study using experimentally infected animals at the ITM experimental station (carrier state experiment). Field samples from two epidemiological different environments in Zambia were collected on filter paper and analysed.

3. **To study the field population structure of *T. parva* in Zambia**. Data on parasite population dynamics are essential to predict possible interactions of different genotypes in the field and future evolution of diversity. The stability in time of genotypes and hence the usefull lifetime of the markers is dependant on the recombination frequencies related to the population structure in the field. Parasites in field samples from both Eastern and Southern Provinces of Zambia were characterised for the three loci (PIM, p104 and p150) and the data were used to define the population structure in the two provinces.
4. **To study dynamics of field population structures after introduction of exotic strain(s) in Zambia**. The multi-genotype characterisation using the three markers was the basis for analysis of interactions of introduced vaccine types with the wild genotypes in both provinces. Several recent cell culture isolates from Southern Province were obtained and field samples from both provinces were collected for multi-genotype data analysis. Allelic frequencies for wildtype and vaccine parasites were determined together with their localisation in the provinces. These data were compaired with characterisation results of cell culture DNA of vaccine stocks and pre-vaccination isolates using the same probes and markers. The cell culture of each vaccine stock was choosen from a passage as closely related in time to the vaccine stabilate.

Several additional loci were surveyed on a subset of the samples to evaluate their potential use for characterising polymorphism among the studied isolates. P67 insert. Primers (ILRI primers nrs 6015 and 6016) encompassing an insert region in the p67 gene were used only on buffalo-derived *T. parva* and *T. lawrencei* isolates to reveal the presence of an insert in the p67 gene. The p67

gene (characterised by Nene *et al.*, 1996) has a very conserved sequence in all cattle-derived *T. parva* stocks studied. In contrast, variable alleles are found in p67 genes of buffalo parasites, with a 80bp insert in the majority of stocks (Collins, 1997).

Linking clones. Six linking clones generated during the construction of a *T. parva* genome map were correlated with polymorphism and primer sequences were available for all six clones (S.P. Morzaria, personal communication). PCR assays were developed for four clones (primers L1-L2; L3-L4; L5-L6 and L7-8) and optimum annealing temperatures determined using a Robotcycler (Stratagene, CA. USA) with an adjustable temperature range. Locus L1-L2 was bridging the fragments 9 and 23 located on chromosome 3; locus L3-L4 was bridging the fragments 33 and 25 located on chromosome 1, locus L5-L6 was bridging the fragments 10 and 27 located on chromosome 4 and locus L7-L8 was bridging the fragments 3 and 31 located on chromosome 3.

A telomeric ORF on chromosome fragment *Sfi*I 1was amplified using the primers (ILRI nrs 4991 and 4992) provided by ILRI.

The ABC cassette gene sequence of *T. parva* Muguga differed from *T. parva* Uganda (Bishop, unpublished results) and intron based primers (ILRI nrs 4989 and 4990) were used to amplify a part of the above locus.

ORF on chromosome 1, *Sfi*I fragment 20. Primers (ILRI nrs 3830 and 3829) amplify a 800 bp fragment in *T. parva* Muguga from the 3' -end of an ORF located on chromosome 1, *Sfi*I fragment 20. Primers ILRI nrs 3831/3829 amplify the full length (~1.5 Kb) of this ORF.

Schizont expressed gene. Primers with ILRI nrs 756 and 757 amplify a schizont expressed protein containing repeated sequences, located on chromosome 1, *Sfi*I fragment 2.

2.2. Materials and methods

2.2.1. Parasite stocks

2.2.1.1. Cell culture-derived parasite stocks

Cell lines were maintained in MEM growth medium enriched with 15% foetal calf serum (Brown, 1977) and passaged three times weekly using 6-well cell culture plates (Gibco, UK). Nine *T. parva* tissue culture isolates were studied from Eastern and Southern Provinces of Zambia before the introduction of the infection and treatment immunisation. These isolates, together with the three components (Muguga, Kiambu5 and Serengeti transformed) of the trivalent vaccine and Mazabuka, a recent Zambian field isolate (1993) from the Southern Province, were cloned by limiting dilution of schizont-infected cell lines and studied over a two year period to assess their genomic stability. Cell lines were cloned by limiting dilution into 96-well plates, seeded with a feeder layer from foetal thymus. *Theileria parva* cell lines were diluted to give a number of one and 0.5 cells per well. The homogeneity of the dilutions was checked by using these two dilution rates in plating and retaining those plates that showed positive well numbers as predicted by the normal Poisson distribution. Wells seeded with the lowest dilution and giving rise to only one growth focus were selected and grown up for further study as clones.

The 13 parasite stocks also formed the basis for the development of molecular characterisation tools for analysis of parasite populations in the field. The recent *in vitro* isolates (1996) Mazabuka1 and Mazabuka24 from Southern Province and Kabwe26 from the Central Province of Zambia were only sequenced for the PIM gene.

DNA extracts of several non-Zambian isolates were obtained from ILRI as additional material to evaluate the allele-based essays. These samples formed the basis to evaluate the value of the developed assays for field investigations. Details of the isolates and stocks used in this study are summerised in Table 7.

2.2.1.2. Field sample collection

Blood samples from clinical cases were collected in the Southern Province, Zambia during May 1996 and from healthy cattle believed to have recovered from ECF outbreaks over the whole Province during February, May and July 1997. Twenty random samples from calves and adults from ten crushpens in Chipata District and from one crushpen in Lundazi District were collected from Eastern Province, Zambia between September and November of 1997. Field blood samples from cattle were collected using EDTA coated Venoject® (Terumo, Belgium) tubes and needles. Blood was transferred to Whatman N°4 filter papers at the laboratory or camp site using the rubber stopper from the blood filled tubes and a filter paper stand. Filter papers were left overnight in a closed cardboard box to keep insects away from the drying bloodspots. They were sealed in plastic bags with silica gel and stored in a fridge or deep freezer within a week of collection awaiting transportion to Antwerp

.

Table 7. Theileria spp used in the study

Theileria parva	Origin	Time	Form	Identity and passages	
Zam2, Chitongo, Zam5,	Zambia, SProvince	May/August, 1982-1983	cell culture isolates, obtained from ILRI	2d passage	Dr. F. Musisi (FAO Project, Lusaka).
Zam6, Zam22, Zam23	Zambia, SProvince	May/August, 1985	cell culture isolates, obtained from ILRI		Dr. F. Musisi (FAO Project, Lusaka).
Mazabuka	Zambia, SProvince	1993	cell culture isolate, obtained from Dr. A. Nambota		Dr. T. Katuda and A. Nambota (Faculty of Veterinary Sciences, University of Zambia, Lusaka)
Katete and Genda	Zambia, EProvince	May/June, 1983	cell culture isolates, obtained from ILRI		Dr. D.Geysen (Andico project, Chipata)
Lundazi	Zambia, EProvince	1983	cell culture isolate from stabilate, ITM	Andico Chipata, stabilate , second passage	Dr. D.Geysen (Andico project, Chipata)
Langa Z424	Zambia, EProvince	March 89	cell culture isolate from stabilate, ILRI	BADC Chipata, stabilate	Dr. L.Lynen (BADC project, Chipata)
Kabwe26	Zambia, Central Province	mai-96	cell culture isolate, obtained from Dr. A. Nambota		Dr. T. Katuda and A. Nambota (Faculty of Veterinary Sciences, University of Zambia, Lusaka)
Mazabuka1	Zambia, SProvince	mai-96	cell culture isolate, obtained from Dr. A. Nambota		Dr. T. Katuda and A. Nambota (Faculty of Veterinary Sciences, University of Zambia, Lusaka)
Mazabuka24	Zambia, SProvince	mai-96	cell culture isolate, obtained from Dr. A. Nambota		Dr. T. Katuda and A. Nambota (Faculty of Veterinary Sciences, University of Zambia, Lusaka)
Muguga (TC646)	Kenya		cell culture isolates, obtained from ILRI	FAO Malawi, stabilate 73, second passage	(Radley et al. 1975c)
Kiambu5 (Z464)	Kenya		cell culture isolates, obtained from ILRI	FAO Malawi, stabilate 68	(Radley et al. 1975c)
Serengeti-transformed	Tanzania		cell culture isolates, obtained from ILRI	FAO Malawi, stabilate 69, first passage	(Radley et al. 1975c)
Muguga	Kenya		shizont DNA extract, obtained from ILRI	ILRI stabilate 3074	
Marikebuni1	Kenya		shizont DNA extract, obtained from ILRI	ILRI stabilate 3014	
Marikebuni2	Kenya		shizont DNA extract, obtained from ILRI	ILRI stabilate 3015	
Marikebuni3	Kenya		shizont DNA extract, obtained from ILRI	cloned line T19.4 infected with ILRI stabilate 3292	
Marikebuni4	Kenya		shizont DNA extract, obtained from ILRI	cloned line G6 infected with ILRI stabilate 3292	
Marikebuni5	Kenya		shizont DNA extract, obtained from ILRI	cloned line BJ253 infected with ILRI stabilate 3292	
Uganda	Uganda		piroplasm DNA extract, obtained from ILRI	ILRI stabilate 3066	
Boleni1	Zimbabwe		shizont DNA extract, obtained from ILRI	ILRI stabilate 3039 from an early passage	
Boleni2 (BOL270)	Zimbabwe		shizont DNA extract, obtained from ILRI	stabilate GU 79-1*	
Boleni3 (BOL320)	Zimbabwe		shizont DNA extract, obtained from ILRI	stabilate GU 79-1*	
Other Theileria species					
T. lawrencei	Kenya		shizont DNA extract, obtained from ILRI	buffalo nr.7014, ILRI isolate G374	
T. taurotragi	Kenya		piroplasm DNA extract, obtained from ILRI	W575-sheep infected line	
T. mutans	Kenya		piroplasm DNA extract, obtained from ILRI	Kipange stock	

* presently used as the vaccine stabilate in Zimbabwe

2.2.1.3. Experimental infections

Experimental *T. parva* infections were conducted under strict tick-free conditions at the experimental station of ITM, near Antwerp. Yearling Friesian calves were inoculated with tick-derived sporozoite stabilates and monitored and sampled daily during the clinical phase of the infections, or on a weekly basis for carrier state studies. *Theileria parva* infected cell-lines were established by *in vitro* infection of PBM, purified using a density gradient (Nycoprep, 1.077 Animal, Nycomed Pharma, Oslo, Norway), with *T. parva* sporozoites (Brown, 1979). *Theileria parva* passages in cattle were performed by inoculation with sporozoite stabilates prepared as described by Cunningham *et al.* (1973). Many Zambian *T. parva* isolates were only available as schizont-infected cell-lines. In order to study infection dynamics and cross-immunity profiles of these isolates, several attempts were made to infect cattle starting with $10^9 - 10^{10}$ culture-derived schizont-infected cells suspended in 20 ml minimal essential medium (MEM).

2.2.2. Study area

Theileria parva is present in two geographically separated areas in Zambia, one in the Eastern Province, and the other in the south-east and central part of the country. The climatic conditions in Zambia have an important influence on the tick population dynamics and hence the transmission possibilities, creating an endemically unstable disease situation in Eastern Province (Billiauw, 1998) and an epidemic situation in Southern Province (Mulumba, 1998). It has been estimated that the tick prevalence rate in Eastern Province ranges between 50-100 AA *R. appendiculatus* ticks per adult animal corresponding with a maximum EIR = 9.6. In the Southern Province tick prevalence rates are between 20 and 50 AA ticks and EIR = 4.8. Buffalo have been reported to pass through Eastern Province occasionally whereas more regular and closer contact is experienced in certain areas in Southern Province.

Various Zambian *T. parva* isolates have been evaluated as sporozoite stabilates in cross-immunity trials in order to determine their suitability for use in the infection and treatment immunisation. In Eastern Province, the infection and treatment immunisation method has been applied using a local stock called Katete and over 120,000 animals have been immunised between 1985 and 1993 (Berkvens *et al.*, 1993). In Southern Province, the trivalent vaccine, referred to as the Muguga cocktail, comprising three stocks, Muguga and Kiambu5 from Kenya and the Serengeti transformed from Tanzania, has been used and 6,000 animals were immunised around Monze from December 1990 to 1992. In addition, the Muguga cocktail was used in about 1,000 animals near Choma between 1986 and 1987 as part of a field trial.

2.2.3. Characterisation of *in vitro* cultured *Theileria parva*

2.2.3.1. DNA extraction protocol for tissue culture cells

The protocol described by Conrad *et al.* (1987) was used to extract DNA from schizont-infected lymphocyte cultures. Cell cultures contained approximately 95% host cell DNA and 5% parasite DNA. Cell pellets were prepared from tissue cultures during the logarithmic phase, using 0.5×10^8 viable *T. parva* infected lymphocytes. Falcon tubes were centrifuged at 200 x g for 15 min at 4°C and washed twice with phosphate buffer solution (PBS). These were stored as dry pellets at -20°C after removal of all fluid with a pipette.

The dry pellets were resuspended in Tris NaCl EDTA (TNE) at 4°C to a final volume of 5 ml and left on the bench to reach room temperature. Sodium dodecyl sulphate (SDS) 10% in ultrapure water (Milli-Q, Millipore, Massachusetts, USA) was used as an extraction buffer and slowly added to the DNA suspension (dropwise over 15 min until the suspension became clear) to a final concentration of 0.5%.

0.05 ml RNase A (Boehringer Mannheim GmbH, Germany) was added to a final concentration of 100 µg ml^{-1}. The suspension was mixed gently and incubated at 37°C for 1-2 hrs. 100 µl Proteinase K (Boehringer Mannheim GmbH, Germany)

was then added to a final concentration of 400 µg ml^{-1} by gently mixing the enzyme in the viscous solution. The suspension was incubated overnight at 50°C.

A standard phenol:chloroform:isoamyl alcohol extraction was used (Maniatis *et al.*, 1982), with a first extraction using pure phenol in order to reduce viscosity. This was followed by a double extraction adding an equal volume phenol:chloroform:isoamyl alcohol (25:24:1) to the sample. The sample was rotated slowly until an emulsion was formed and centrifuged at 1600 x g for 3 min at room temperature, which separated the different layers and the supernatant (aqueous phase = DNA phase) was collected using a 5 ml pipette. This was repeated, usually twice, or until no protein was visible at the interface of the organic/aqueous phases. A last extraction was performed using neat chloroform or chloroform:isoamyl alcohol (24:1) to remove all traces of phenol.

The DNA extracts were collected and subjected to an alcohol precipitation on ice with sodium acetate (NaAc) by adding ± 600 µl NaAc 3M and 1.8 ml 100% ethanol (1:3 ratio) at 0°C and mixing. This was kept overnight at -20°C. The DNA plug was recovered from the 100% alcohol and transferred, after a short submersion in 70% ethanol, to an Eppendorf tube. After centrifugation at 1600-5000 x g for 10 min at 4°C, the supernatant was removed by carefully pouring, without disturbing the invisible DNA pellet. The tube was filled again with 70% ethanol at room temperature and centrifuged at 1600 x g for 15 min at 4°C. The supernatant was removed and the washing was repeated once more. The last supernatant fluid was removed with the use of a pipette. The sample was dried for 1-2 hrs on the bench and the DNA pellet was dissolved in 1 ml sterile Tris-EDTA (TE). The sample was incubated at 55°C for 2-3 hrs and left overnight at 4°C to dissolve all DNA to obtain a good homogenous mixture. The optical density was measured using a spectrophotometer (Model 24, Beckmann, CA, USA) and the DNA concentration was calculated. Typical yields of DNA obtained with this protocol ranged between 0.8-3 µg/µl DNA.

Various solutions and buffers:

PBS
8 gr NaCl, 0.2 gr KCl, 1.44 gr Na_2HPO4 and 0.24 gr KH_2PO4 in 1 liter aqua distillata and adjusted to pH 7.4 with HCl.
1 M Tris pH7.5
121.14 gr tris pH 7.5 in 1litre ultrapure water (Milli-Q, Millipore, Massachusetts, USA) used at a 0.2M concentration.

TNE
Concentration of Tris-HCl, NaCl and EDTA as follows:
25mM Tris-HCl (pH8,0)
100mM NaCl
5mM EDTA.

TE, pH 7.6
Concentration of Tris-HCl and EDTA as follows:
10mM Tris-HCl and 1mM EDTA.

TAE 1x
0.04M Tris-acetate and 0.001M EDTA

TBE 0.5x
0.045M Tris-borate and 0.001M EDTA

SSC 20x
175.3 g NaCl and 88.2 g sodium citrate in 1000 ml aqua distillata. (pH 7.0)

RNase A
solution of 10 µg ml^{-1} TE prepared by boiling for 10 min at 93°C to free DNase, and kept aliquoted at -20°C.

Proteinase K
solution of 20 µg ml^{-1} in double distilled water, stored at -20°C.

Sodium acetate
0.3M with pH 5.2.

2.2.3.2. DNA digestion, gel electrophoresis and Southern blotting

*Eco*RI restriction enzyme digestion was performed according to the manufacturer's specifications (Gibco, UK) using 10 units μg^{-1} DNA. The digestion mix consisted of 10-20 µg of schizont DNA in 80-100 µl total volume. The reaction was left overnight in a water bath at 37°C. A volume of 30 µl of the digested sample was mixed with 6 µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll in water) and transferred onto a 0.8% agarose gel. A *lambda*-DNA ladder (2-23 kb range) marker (*Lambda* DNA/ *Hind* III Digest, Eurogentec, Belgium) was included on the gel to size DNA positions. The DNA fragments were separated by horizontal electrophoresis in Tris Acetate EDTA

(TAE) buffer at 20 V for 16-18 hrs. In some cases field inversion gel electrophoresis (FIGE) using the pulsed field switch E791 apparatus (Consort, Belgium) was performed in order to obtain a better separation. The settings used were Pt1 (run time of normal direction) = 2.1 sec; Pt2 (run time of inverse direction) = 0.7 sec and Pt3 (Interval between field inversions) = 0.1. The gel was stained for 25 min in ethidium bromide and destained in double distilled water for 1-1.5 hrs and photographed under UV illumination. Transfer of the restricted fragments from the gel onto nylon filters was done by Southern blotting in sodium salt citrate buffer 20x (SSC 20x) after appropriate treatment of the gel and nylon filter as described by Maniatis *et al.* (1982). The filters were dried for 1 hr, marked and packed in foil. The DNA side was exposed to UV light for 3-5 min before storage at 4°C.

Solutions used were made as follows:
Gel treatment by acid depurination was performed for a maximum of 30 min in 0.25N HCl under slow shaking followed by basic denaturation (1-1.5 hrs) in 1.5M NaCl and 0.5M NaOH under slow shaking. The treatment was stopped by neutralising the gel during 1 hr in 1.5M NaCl and 0.5M Tris pH8 under normal shaking. Ethidium bromide was used at a concentration of 1 µg ml^{-1} solution.

2.2.3.3. DNA radiolabelling and hybridisation

Probes were radiolabelled with $^{[32]}P$ dCTP (3000 Ci mmol-1; Amersham International PLC, Little Chalfont, UK) by a nick translation kit (Stratagene, La Jolla, CA, USA) to $0.5^{-1}x10^{8}$ cpm μg^{-1} activity, using a random priming method (Feinberg and Vogelstein, 1984). The radio-labelled DNA was cleaned by alcohol precipitation, resuspended in TE and stored at -20°C.

Filters were incubated and shaken continuously at 65°C for at least 2 hrs in a pre-hybridisation solution containing 6 x SSC, 10 x Denhardt's solution, 0.05% SDS and 50 µl of denaturated (boiled for 3 min) salmon's sperm solution. Radio-labelled probe was denaturated by boiling during 10 min and added to the pre-hybridisation solution at a concentration of 5 x 10^{5} cpm ml^{-1}. Hybridisation was

performed in a shaking water bath at 65°C for at least 18 hrs. The hybridised filters were washed twice in 2 x SSC at 65°C for 15 min and twice for 30 min at 65°C using 1 x SSC with the addition of 0.1% SDS (normal stringency wash). Filters were then dried and exposed to Kodak RX 100 film with intensifying screens at - 80°C for 1-21 days. Filters were reused after washing in NaOH and 0.1 x SSC with 0.1% SDS and 0.2M Tris-HCl and checking for remnant radioactivity.

Solutions used were made as follows:

10 x Denhardt's solution was prepared as described by Maniatis *et al.* (1982). 50 µl salmon sperm (aliquots of 10 mg ml^{-1}) was boiled for 3 min at 100°C, transferred to ice for immediate denaturation. Probed filters were washed in a mixture of 0.2M Tris pH 7,5 and 0.4M NaOH and checked for remnant radio-activity before use in hybridisation with a new probe.

2.2.3.4. DNA probes used for typing

The DNA probes were obtained from ILRI and there location in the *T. parva* genome is shown in Figure 12 (page 104).

(i) The telomere probe used is the pTpUtel, which consisted of a 1.74 kb telomeric fragment, isolated from *T. parva* Uganda and is present on all eight telomeres of the four chromosomes (Allsopp and Allsopp, 1988).

(ii) The Tpr1 probe is a 623 bp fragment from the Tpr1 locus, isolated from *T. parva* Muguga and cloned in pUC19 (Sohanpal *et al.,* 1995). This locus is centromeric on chromosome three and covers approximately 2% of the total genome. The Tpr1 probe hybridises with multiple sequences present at this locus.

(iii) The LA6 probe used is based on shizont cDNA cloned in pcDM8 and has been shown to hybridise to multiple genomic copies in *T. parva* DNA (Bishop *et al.,* 1993a). It spans a proline and methionine rich domain, present on chromosomes two and three.

(iv) The Minisatellite probe (TpMS 111) reacts with multiple dispersed loci on all chromosomes (Bishop *et al.*, 1998). Both the LA6 and TpMS 111 probes were prepared from a PCR product.

2.2.4. Characterisation of field samples

2.2.4.1. Extraction of field samples

A Chelex based extraction protocol for blood samples, described by Walsh *et al.* (1991), was adapted at ITM (de Almeida *et al.,* 1997) for PCR based trypanosomiosis diagnosis on samples from blood impregnated filter paper. The adaptation consisted in an extended elution step and treatment in 1% Chelex. A similar method has been used for *Plasmodium* extraction in blood derived from filter papers. (Kain and Lanar, 1991; Wooden *et al.,* 1993).

Two filter paper discs from blood spots (each disc representing 5 µl blood), cut using a 6 mm perforator, were dropped into a 1.5 ml Eppendorf tube, containing 1 ml of ultrapure water. The perforator was flame-heated between each sample. The discs were hand shaken vigorously twice using a 15 min interval between each agitation. The discs were then removed and the sample was centrifuged at 11000 x g for 10 min. A volume of 800-900 µl supernatant was removed without disturbing the pellet. Two hundred µl of a 1% Chelex-100 resin suspension (Bio-Rad, Hercules, CA) in ultrapure water was added to each tube and incubated for 30 min at 56°C. The tubes were transferred to a boiling water bath and left to boil for 8 min.

The samples were agitated on a vortex mixer at maximum speed for 2 min and centrifuged at 11000 x g for 5 min. Three hundred µl supernatant was transferred into a new 1.5 ml Eppendorf tube and stored at -20°C until used. Five µl supernatants per 25 µl PCR reaction was used. This corresponds to a 1/30 dilution of the initial blood sample, giving an equivalent of 0.17 µl blood used per PCR reaction.

The detection threshold was evaluated by using serial dilutions of a blood sample of known parasitaemia and calculating the numbers of parasites in the lowest dilution producing a positive PCR result using the PIM, p104 and p150

assays. The dilutions were prepared with uninfected bovine blood each spotted on filterpaper and further extracted. The sensitivity of the PCR assays was tested on filter paper samples taken weekly from 19 known *T. parva* positive animals at the experimental station of ITM. Furthermore, blood samples from two animals were analysed on a regular basis over a two year period to assess the carrier state dynamics.

2.2.4.2. Amplification of *Theileria parva* field isolates

Extracted *T. parva* genomic DNA was used as a template in PCR amplification. The reactions were carried out in a total volume of 25 µl. Each reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 20 pmoles of each primer, 0.5 U Taq polymerase enzyme (Goldstar, Eurogentec) and 5 µl of a DNA solution (at a concentration of 10 ng μl^{-1} in the case of cell culture extracts). Each mixture was overlaid with 50 µl of fine neutral mineral oil (Sigma) and placed on a heating block set at 90°C for PCR amplification (PTC-100, MJ Research, Mass. USA; Robotcycler, Stratagene, CA. USA or Thermolyne, Barnstead, Iowa, USA). In case of semi-nested runs, 0.5 µl of amplification product from the first run was added through the oil layer to the semi-nested mix at 82°C (hot start principle), containing the same ingredients and concentrations, except that 0.3 U Taq was used. The amplification programme was as follows. Step 1: 94°C for 4 min; Step 2: 94°C for 1 min; Step 3: annealing temperature for 2 min; Step 4: extension temperature of 72°C for 2 min; Step 2-4 were repeated for 39 times in case of first run and 24 times in case of the semi-nested run. Step 5 was a final extension phase at 72°C for 8 min. The amplification was terminated by reducing the temperature to 20°C or 6°C.

The mixtures were examined for the presence of DNA fragments by loading 5 µl of each reaction mixed with 2 µl of 5 x loading buffer onto 2 % agarose mini gels (Muped II, Eurogentec, Belgium). A 100bp DNA ladder (MBI Fermentas, Lithuania) was included in every gel. The samples were run for 20 min at 100 V, stained in ethidium bromide for 20 min, washed under running tap water and

photographed under UV illumination. For further typing of the fragments, heteroduplex, SSCP or RFLP based methods were used.

p104 assay

Primers were designed encompassing the polymorphic region, giving a 1000 bp amplification product. First run primers p104/2 and p104 Rv were used with an annealing temperature of 58°C. Nested run primers p104/1 and p104/2 were used with an annealing temperature of 60°C. Nested products were digested with *Alu*I enzyme and RFLP profiles determined on 10% PA gels.

Primers	p104/1 (ILRI 759)	: TAAGATGCCGACTATTAATGACACC
	p104/2 (ILRI 755)	: CCGTTTGATCCATCATTCAAGG
	p104Rv (ITM)	: GCATAATTCAAATGTCCGGGTAT

p150 assay

Primers were designed to encompass the entire polymorphic region, amplifying a region of approximately 940 bp. First run primers Np150F and Np150R were used with an annealing temperature of 64°C. Nested run primers Np150nF and Np150R were used with an annealing temperature of 60°C

Nested products were digested with *Sau*3AI enzyme and RFLP profiles determined on 10% PA gels.

Primers	p150R (ILRI)	: TTACCATCTTCACCGCGAAC
	p150F (ILRI)	: GATATTCCTTTACTTGCTCGAC
	p150nF (ITM)	: CGACTTGAAGAAGAAGATTACAGT

PIM assay

The gene reveals important size polymorphism and primers were used which annealed in the conserved end regions. First run primers PIMF and PIMR were used with an annealing temperature of 62°C. Nested run primers PIMnF and PIMR were used with an annealing temperature of 58°C. Nested products were digested with *Bcl*I enzyme and RFLP profiles determined on 10% PA gels.

Primers	PIMF (ITM)	: ATTCCACTGGTTCTTCCGATGT
	PIMR (ITM)	: CAACCGTGGAATGGCGTATGTT
	PIMnF(ITM)	: AACACAAGTTGATACTGAAT

P32 assay

This was not used as a routine assay. First run primers P32F and P32R were used with an annealing temperature of 55°C. Nested run primers P32FTot and P32R were used with an annealing temperature of 61°C. Products were analysed using the SSCP technique.

T. parva specific primers:

P32F (ILRI)	: GTTAATGCCGCAAAAGAAGAAGAG
P32R (ILRI)	: AAGGTTGTAAAAGACTGAAGCGGC
p32FTot (ITM)	: ATTATTTGAGATGTTGTCCA

Theileria taurotragi specific primers were not used as a routine on all samples but were used occasionally when confounding infections were suspected or outbreak suspected samples showed negative on *T. parva* PCR.

Theileria taurotragi specific primers:

TAU.F (ILRI)	: AAACATGGTGAAGAGGAATGG
TAU.R (ILRI)	: GTAGTCAAGTCTTACGACTGC

Tpr1 amplification

This locus was not routinely amplified. Tpr1 primers used for this region and the amplification programme were described by Bishop *et al.* (1992). Samples were analysed for size polymorphism on a 10% PA gel.

18S assay

This was not used as a routine assay. First run primers 18SF and 18SR were used with an annealing temperature of 54°C. Nested run primers 18SF and 18SNR were used with an annealing temperature of 60°C. Nested products were digested with Msp1 enzyme and RFLP profiles determined on 10% PA gels.

18SF (ITM)	: CGTTTTTACATGGATAACCGTGCTA
18SR (ITM)	: AACTGACGACCTCCAATCTCTAGTC
18SNR (ITM)	: GGCATTGTTTATGGTTAGGA

2.2.4.3. Characterisation methods

2.2.4.3.1. Heteroduplex method

This method reveals sequence differences when a mixture of single stranded DNA (ssDNA) of two samples is allowed to re-anneal before separation on a 5% polyacrylamide (PA) gel. This method has been adapted to HIV-1 strain classification by Delwart *et al.* (1995) and the same method has been adapted at ITM for screening PCR products of different *T. parva* gene loci. Heteroduplexes were generated by using 5 µl of PCR product from the test sample with 5 µl of PCR product from the reference stock and 1.1 µl of 10 x heteroduplex annealing buffer. The mixture was heated to 94°C for 2 min in a water bath. The tubes were cooled rapidly by transferring them into wet ice. The samples were loaded onto a 5% PA gel with 1 µl of a 5 x Ficoll-Orange loading buffer. Electrophoresis was done in a 0.5 x TBE buffer over 6 hrs at 200V using a horizontal device (Farmacia, Uppsala, Sweden) and gels were stained using ethidium bromide.

Solutions used:

10 x heteroduplex annealing buffer: 1M NaCl, 100mM Tris (pH 7.8) and 20 mM EDTA solution.

5 x Ficoll-Orange loading buffer: 25% Ficoll and 1% Orange G solution in water.

2.2.4.3.2. The SSCP method

This method demonstrates conformation based changes in single stranded DNA strands due to single basepair differences in PCR-generated products (Orita *et al.*, 1989). The sensitivity of this method is dependent on the fragment size of the PCR product and the temperature used during electrophoresis. Best results are obtained using fragments in the 150-400 bp range. Longer fragments might not reveal single basepair differences but would still give a fair indication of heterogeneity.

PCR products were denaturated according to different protocols and 4 - 8 µl loaded into pre-run 10% PA gels. The gels were run in 1x TBE buffer over 18 hrs at 100V on a vertical Mighty Small II electrophoresis device (Hoeffner, UK) at 18°C.

Standard protocol

Samples were diluted in a formamide mix and denaturated by heating in a water bath at 95°C during 5 min. Samples were directly transferred into ice water and left for another 10 min. A total sample volume of 6 µl was loaded and 2 µl of a diluted *lambda* ladder was included as marker at the extreme ends of the gel.

Solutions used:

Formamide mix consists of a solution of 95% formamide (de-ionised), 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol.

Loading buffer: Solution of 0.25% bromophenol blue, 0.25% xylene cyanol and 15 % Ficoll in water.

In a preliminary study different protocols were analysed to determine optimal denaturation and separation conditions. The denaturation mixtures consisted usually of a loading buffer and products acting on the stability of double or single stranded DNA. NaOH interferes with H-bridges, destabilising DNA structures, SDS would protect SS-strand formation and EDTA interferes with Mg^{++} iones.

Protocol 1: Denaturation was achieved by adding 1µl of an 0.5M NaOH/10mM EDTA solution to 9 µl PCR product and heating at 42°C for 5 min and cooling on ice for another 5 min. Loading buffer was made of 0.05% xylene cyanol and 0.05% bromophenol blue in 95% formamide.

Protocol 2: Denaturation was achieved by adding 10 µl of an 0.5M NaOH/1mM EDTA solution to 3µl PCR product and heating at 50°C for 10 min and cooling on ice for another 10 min. Loading buffer was made of 0.05% xylene cyanol and 0.05% bromophenol blue in 15 % Ficoll.

Protocol 3: Denaturation was achieved by adding 3 µl of an 0.05% xylene cyanol, 0.05% bromophenol blue and 20mM EDTA solution in 95% formamide to 5 µl PCR product and heating at 95°C for 3 min and cooling on ice for another 10 min.

Protocol 4: Denaturation was achieved by adding 20 µl of a 10 mM EDTA, 0.1% sodium dodecyl sulphate solution to 5 µl PCR product. 3 µl of this was mixed with 3µl loading buffer and heated at 94°C for 4 min.

Samples were immediately loaded onto:

A. non-denaturing 10% TBE-PA minigel (1 mm thick) under constant voltage or power (18 Watt) with 0.6 x TBE buffer for 338 Vh.

B. non-denaturing 10% TBE-PA minigel (1 mm thick) under constant voltage with 0.8x glycerol tolerant buffer with 5% glycerol for 720 Vh.

PA gels were made with 0.6xTBE buffer and 25% (vol/vol) 2 x MDE (Mutation Detection Enhancement) stock solution (PAGE Hydrolink), 0.25 M ammonium persulphate and 0.1% TEMED. The stock solution 1 x TBE consisted of 50 mM Tris (pH 8.3), 50 mM boric acid and 10 mM EDTA. Prepared gels were individually packed and stored at 6°C prior to use.

2.2.4.3.3. RFLP-PCR method

This method reveals DNA fragment size polymorphism generated following digestion with endonuclease. When sequence data were available, identification of suitable restriction enzymes was done with the aid of a computer programme (GeneJockeyII). Restriction enzyme digestion was done according to the manufacturer's specifications (Gibco, UK using 10 units μg^{-1} DNA (0.6 U μl^{-1} PCR product) on 5 µl of amplified DNA in 15 µl total volume. The reaction was left overnight in a water bath at the specified temperature. Six µl of restricted sample mixed with 2 µl loading buffer was transferred onto a 10% PA gel together with a 100bp DNA ladder (MBI Fermentas, Lithuania) for fragment size determination. DNA fragments were separated by horizontal electrophoresis in 0.5x TAE buffer at 100 V for 2.5 hrs. The gel was stained using a commercial silver staining kit (Silver staining kit DNA plusone, Pharmacia Biotech, Uppsala, Sweden) and mounted for storage.

2.2.4.3.4. Cloning of PCR products

Normal primers were used for cloning except when pUC18 was used as the plasmid vector (Pharmacia Biotech, Uppsala, Sweden) when the PCR reaction was performed using *Eco*RI and *Bam*HI adapted primers. The PCR products were purified using the Promega Wizard DNA clean up kit (Promega Corp. Madison, WI, USA) before ligation into a plasmid vector (usually pUC18 at 50 ng

μl^{-1}). Ligation was done overnight with T4 DNA ligase (8 Units) at a vector: PCR product rate of 1:2 in maximum 20 µl total volume at16°C.

E.coli competent cells (JM109, Promega, USA) were transformed by mixing with the cloned plasmid and subsequent heat-shock treatment according to the manufacturer's instructions, or 200-400 µl of an exponentially growing competent cell suspension was transferred using a cold chilled tip to an Eppendorf tube, cloned plasmid added, mixed by swirling gently and stored on ice for 30 min. Tubes were placed in a 42°C circulating water bath for exactly 90 seconds and transferred rapidly to an icebath for 1-2 min. Finally, 1.6 ml of LB was added and the tubes were transferred for another 45 min to the incubator.

The cell suspensions were plated in two different concentrations on special LB agars with the necessary controls (restriction, ligation, DNA and vector). They were left for 16-24 hrs in the incubator at 37°C. Pure white colonies were screened by PCR for positive sequences and colonies were subcultured for plasmid extraction or for storage as glycerinated stabilates.
The cloning of the PCR products from Zam22, Zam23 and Chitongo stocks was done using a TA pCR®2.1-TOPO vector with topomerase based incorporation of the PCR product according to the manufacturer's manual (using the TOPO TA Cloning kit of Invitrogen, USA).

LB agar was made by adding 5g Bacto®-Tryptone, 2.5g Bacto®-Yeast Extract and 2.5g NaCl to 500 ml aqua dist. Special LB agar was prepared by adding 2.5 ml ampicillin solution of 20 mg ml^{-1}, 1 ml X-gal solution (1 ml 5-bromo-4-chloro-3indolyl-ß-D-galactoside 2% w/v in dimethylformamide and 2.5 ml IPTG solution (3 x 850 µl isopropyl-ß-D-thiogalactoside, 100 mM l^{-1}) to 500 ml LB agar.

2.2.4.3.5. Sequencing of cloned PCR products

Glycerinated stabilates were sent to the Sequencing Department of Eurogentec, Belgium, for sequencing. The sequence was determined by the dideoxy chain termination method on an ABI PRISM™ Genetic Analyser (Perkin Elmer ABI, CA, USA) during a single run starting at both ends for about 500bp.

Sequence data were received via e-mail and a hard copy was received by post. The sequences of Zam5 and the short Katete PIM alleles were obtained from two recombinant clones each. PIM sequences from two different Mazabuka isolates and one Central Province isolate (Kabwe26) were also determined (ITM for Maz1 and ILRI for Maz24 and Kabwe26).

2.2.4.3.6. Cloning and sequencing of PIM

Ten sequences were obtained by determining the nucleotide succession of cloned PCR products from the Katete and Genda stocks from the Eastern Province, and Chitongo, Zam22, Zam23, Zam5 and Mazabuka isolates from the Southern Province. Sequences were aligned by using GeneJockeyII software (Biosoft, Cambridge, UK) and finalised by hand using SeqVu 1.0.1. software. Nucleotide and deduced amino-acid sequences and restriction enzyme sites were analysed using GeneJockeyII software (Biosoft, Cambridge, UK).

3. Results

3.1. Protocols and methods of analysis

3.1.1 Cloning results

Forty nine clonal populations were derived from nine stocks using the limiting dilution technique (Katete: 5 clones; Genda: 8 clones; Zam3: 3 clones; Zam5: 5 clones; Zam22: 4 clones; Zam23: 4 clones; Mazabuka: 13 clones; Muguga: 4 clones and Kiambu: 3 clones). Most of the *T. parva* cell culture isolates contained minor subpopulations as shown by the different but related profiles when comparing Southern blot results of parent isolates with their clones. The cloning method based on limiting dilution is considered adequate for molecular studies although it is theoretically possible that a lymphocyte can become infected by more than one sporozoite (Fawcett *et al.*, 1982). Morzaria *et al.* (1995) described a 3-step method to generate *T. parva* clones in the true genetic sense for use in studies of strain specific immune responses. The clone profiles did not show any variation in time while the parent profiles did so. The results confirm that the limiting dilution method is adequate to generate clones.

3.1.2. PCR sensitivity and specificity

The modified extraction method (de Almeida *et al.*, 1997) using blood on filter paper samples gave very good results. In a comparison between normal and semi-nested amplification of 68 field samples from clinical cases in Southern Province, the semi-nested technique using the p104 primers gave 80% positive amplification against 60% positives and 20% doubtful (characterised by a very weak amplification band on agarose gel) results using a single PCR amplification reaction using p104Rv and p104/2 primers.

The detection threshold was determined for the semi-nested PCR assays based on PIM, p104, p150 and 18S genes. The p150 assay was found to be the most sensitive detecting between 3 and 30 parasites per reaction. This corresponds to a detection level of around 0.0003% parasitaemia.

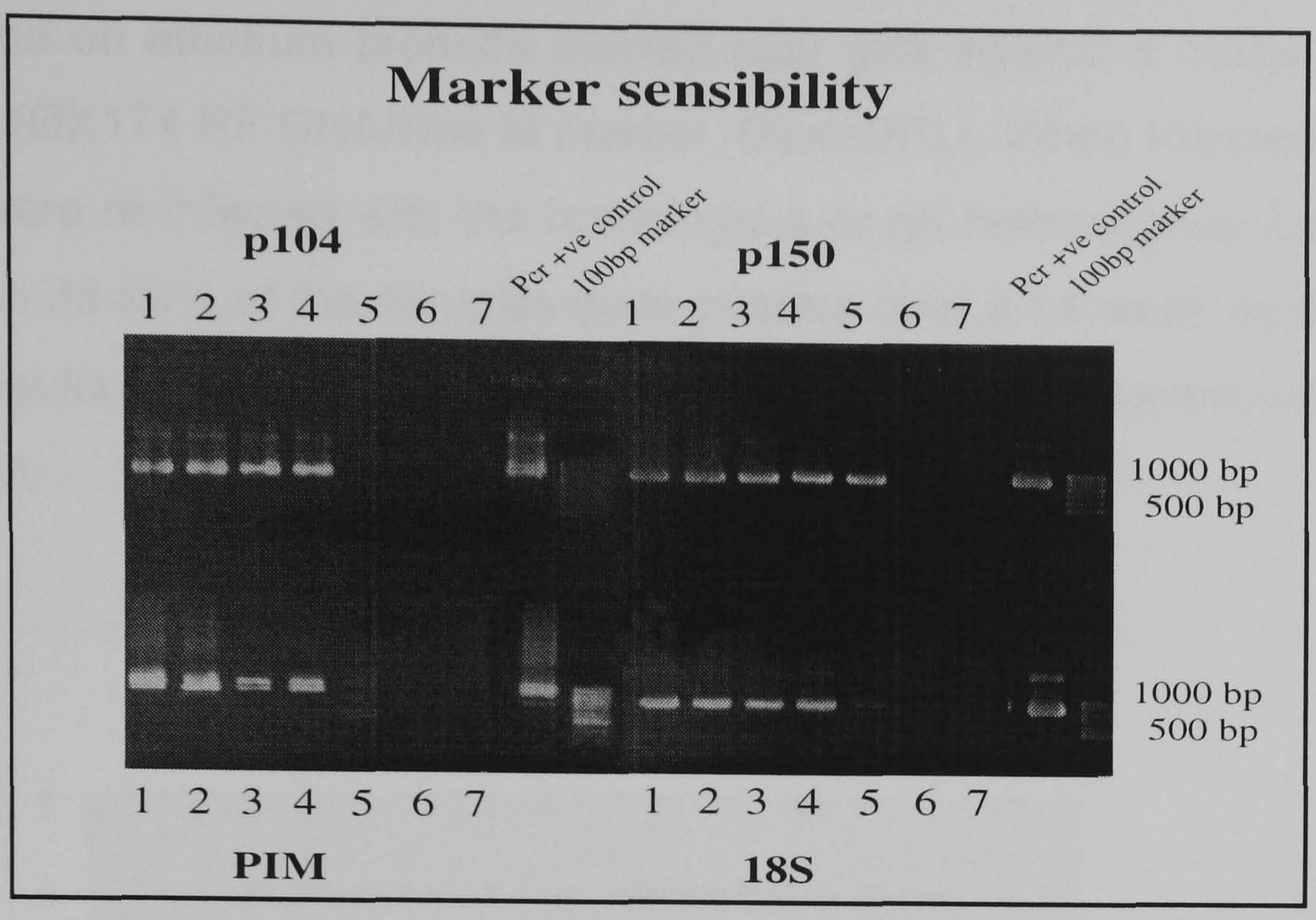


Fig. 13 PCR results of different 10 fold dilutions of a blood sample amplified in a nested reaction using the p104, p150, PIM and 18S primers. Results on a 2% agarose gel, stained with ethidium bromide. 1=undiluted sample; 2=0.1 diluted sample; 3=0.01 diluted sample; 4=0.001 diluted sample; 5=0.0001 diluted sample; 6=0.00001 diluted sample and 7=10^{-6} diluted sample

The PIM, p104 and 18S primers produced detectable fragments when minimum 30 parasites were present as template for the PCR reaction (Fig. 13). The results from the weekly samples of the two chronically infected animals (Nrs 3858 and 3856) and additional results from animals infected with another stock or after challenge with a homologous (Nrs 1772 and 1330) or heterologous stock (Nr 1320) are also presented (Table 8).

Animal Nr.	Stock	Weeks	+ve	% Weeks	Total
Single infection					**53%**
3858	Katete	48	24	50%	
3856	Katete	48	38	79%	
1330	Chitongo	14	4	29%	
Challenge					**69%**
1772	Homologous, Katete	14	11	79%	
1330	Homologous, Chitongo	9	8	89%	
1320	Chitongo challenged with Katete	21	8	38%	

Table 8. PCR results of weekly samples from a longitudinal study of experimental *T. parva* infections. Weeks: number of weeks sampled; +ve: number of weeks when samples were found positive; % Weeks: % of weeks animal showed positief PCR results.

In the study of two carrier animals (Figs. 14-15) approximately 65% of the weekly bloodspot filter paper samples were found positive on PCR analysis using the

p104 assay. The Y-axis relates to the strength of the amplification signal as assessed on ethidium bromide stained agar gels against a 100bp quantitative marker (ØX174 RF DNA/Hae III marker, GibcoBRL). When infected animals (n=3) were re-infected with the homologous or an heterologous Zambian stock, between 38-89% of the samples were positive over a 14 week period. When the PCR results were plotted over time no correlation was apparent with length of infection.

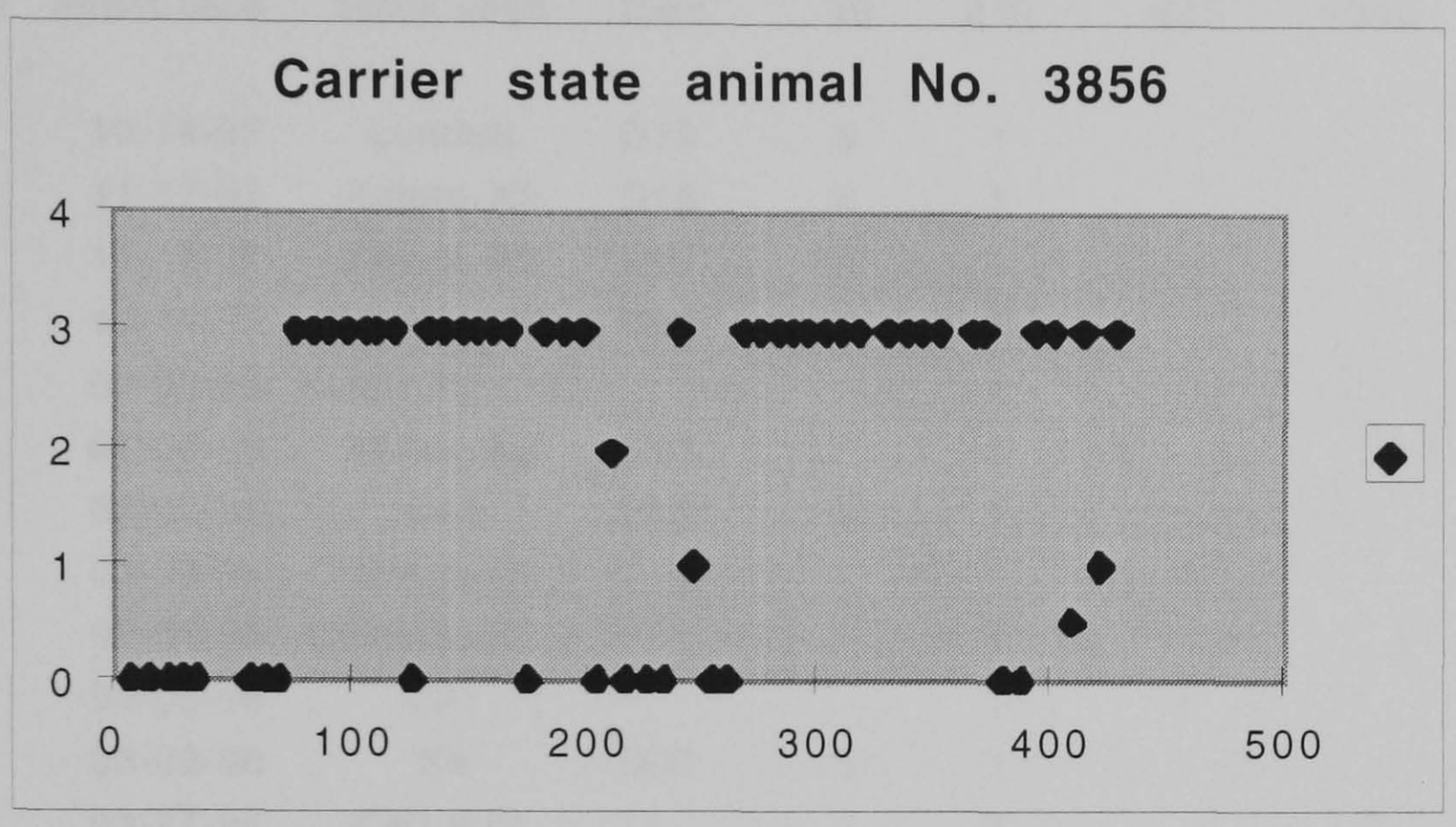


Fig 14. PCR results from a carrier animal over a 430 day period after heterologous challenge. Dots indicate weeks of blood sample collections. Y-axis relates to the strength of the amplification signal with 0 as no signal found (negative). X-axis indicate days after infection.

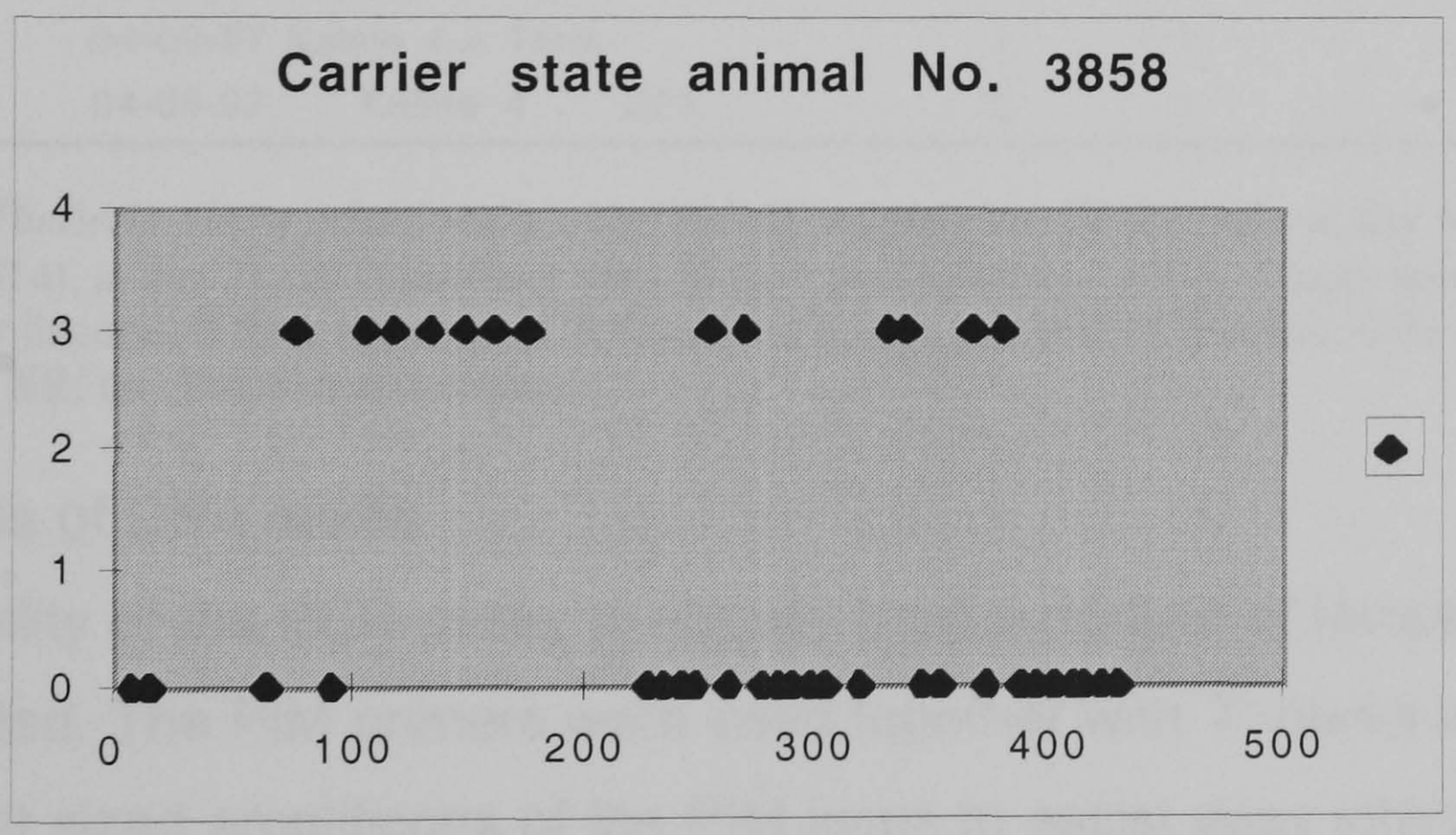


Fig.15. PCR results from a carrier animal over a 430 day period after heterologous challenge. Dots indicate weeks of blood sample collections. Y-axis relates to the strength of the amplification signal with 0 as no signal found (negative). X-axis indicate days after infection.

When samples (n=394) from the field were analysed using the 18S assay more than 99% of these resulted in a PCR product of the expected size. Further

characterisation of these by RFLP using *Msp*I gave recognisable Theilerial profiles corresponding to multi or single species infections.

When bloodspot filter paper samples from 19 experimentally infected animals were amplified using the p104 assay all animals became positive on PCR between days 8 and 21. As has been reported above, blood from known carrier animals from the experimental stable gave regular PCR amplification.

Animal nr	infect.date	Stock used	Died	d8	d14	d21	>30d	Clinical reactions
3279	10-14-97	Lundazi	D18	0	1			died
3295	11-17-97	Katete K3	D18	0	1			died
654	11-17-97	Katete K3	D22	0	1			died
3322	10-14-97	Chitong S7/2	D20	1	1			died
9719	01-09-98	Katete K2 LYO			1			NR
4863	01-09-98	Katete K2	D23		1			died
2297	03-03-98	CA1	D19	0	1			died
2299	03-03-98	Chitongo S7,2			1	1		Clin
5195	03-03-98	Chitongo S7,2			1	1		Clin
8565	03-05-98	CA1			1	1		Clin
1843	03-03-98	K4	D20	0	1			died
9538	03-27-97	CH1 0,01			1		0	NR
1321	03-27-97	CH1 1/1	D23		1	1		died
3	06-11-97	K4 1/3			0	1		Clin
4	06-11-97	K4 1/3	D35		0	1		died
5907	04-09-97	Katete V1			1	1		Clin
7195	04-09-97	Katete 4 + Terra			1	1		Clin
7198	04-09-97	Katete 4 + Terra			1	1		Clin
6434	04-03-97	Katete 4	D21		0	1		died

Table 9 *Theileria parva* infections in experimental animals and PCR results at day 8 post infection (d8), at day 14 (d14), at day 21 (d21) and later than 30 days post infection (>30d). Empty boxes representing no sample collection, 0 for a negative PCR result and 1 for a positive PCR result. Clin indicates a clinical reaction; NR: no clinical reaction seen.

Analysis of DNA mixes

The ability of the PCR assay to amplify from a mixture of templates was also evaluated. The PIM primers were used together with *T. parva* isolates giving different sized amplificons of the PIM locus to assist easy interpretation of the results. The *T. parva* Kat4 (a fourth passage of the *T. parva* Katete isolate), Zam5 and Muguga stocks were used in the mixture experiment. Three different DNA mixtures (5:5; 3:7 and 1:9 ratio) of two *T. parva* isolates were amplified using the PIM assay and results were compared. All genotypes were amplified although not to the same extent (Fig. 16).

The Kat4 amplification was suppressed in the 5:5 mix except when mixed with Muguga where Muguga was more suppressed than Kat4. This suppression was eliminated when the ratio was raised to 1:9. In the remaining mixes, the Kat4 suppression in the Zam5 mix was eliminated when the mix was raised to 7:3.

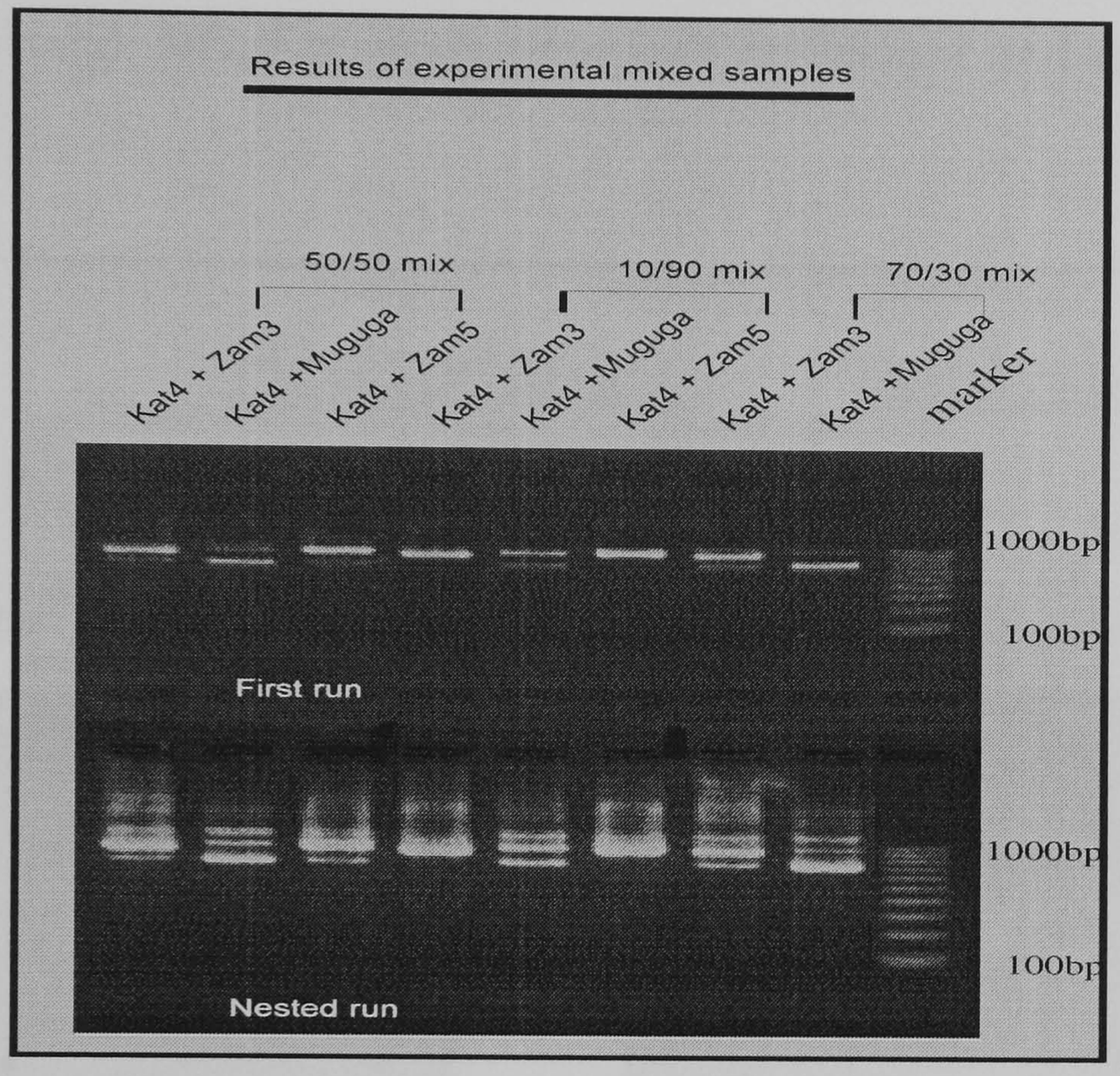


Fig.16. Comparison of first and nested PCR results of experimentally mixed DNA extracts from two cloned *T. parva* isolates showing different genotypes. The Kat4 product is 700bp long while the others are 1000bp long. The band of 1300 bp in the nested run is an aspecific product.

3.1.3. Methods of sample analysis

Heteroduplex (HD) analysis (Fig.17) could only differentiate among various isolates when using the PIM locus showing marked polymorphism. Good differentiation was only obtained on p104 or p150 locus amplified products when the DNA originated from buffalo-derived stocks. The extent of difference is related to the location of the heteroduplex bands on the gel, higher bands disclosing greater differences between the two samples tested. The polymorphism among the PIM products was of such extent that the heteroduplexes remained close to the top. HD results confirmed the outstanding polymorphism found at the PIM locus followed by a moderate polymorphism

among the buffalo-derived Zam5 and Boleni stocks using the p150 locus and a slight difference for these stocks using the p104 locus.

SSCP based analysis generated different profiles among various stocks for most loci but results were difficult to compare between different gels. A major problem was a 'smiling profile' in the gels (differential migration of samples from the sides towards the middle of the gel, giving a curved profile), especially interfering with characterisation of isolates showing minor differences in profile. On the other hand, RFLP analysis produced consistent and reproducible profiles.

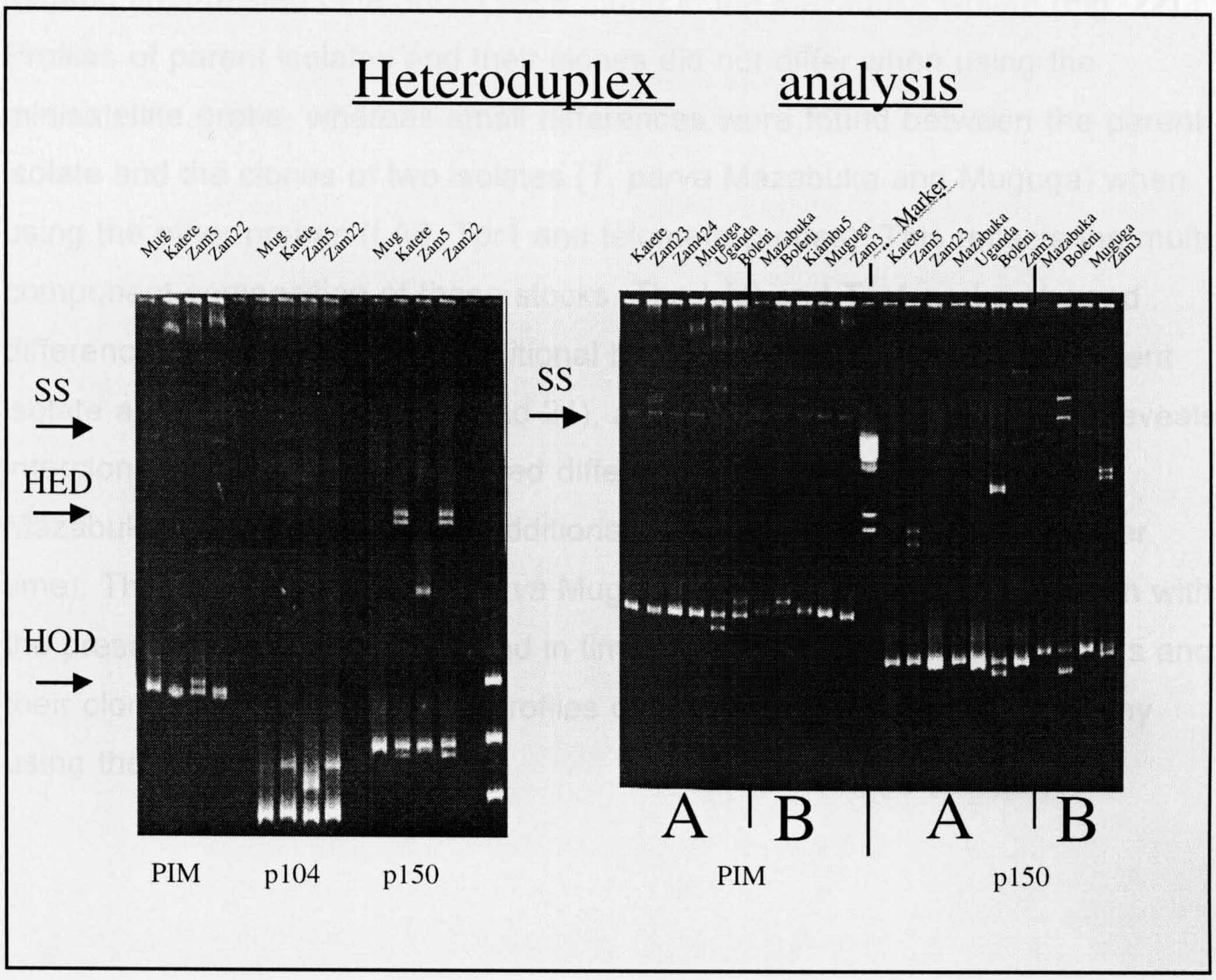


Fig. 17. Heteroduplex analysis of several loci of different stocks. Left picture shows results of several stocks against a Muguga reference and right picture against a Katete (A) and a Mazabuka (B) reference. Homoduplexes of both components of the sample are located towards the bottom of the gel, heteroduplexes are migrating slower and located in the middle of the gel. Faint bands consist of single stranded DNA. SS = SingleStranded DNA. HED = Heteroduplex. HOD = homoduplex DNA.

3.2.' In vitro' studies on genomic stability

Four different *T. parva* probes were hybridized to Southern blots of *Eco*RI-digested schizont DNAs from 13 *T. parva* infected cell cultures and their derived clones. The Southern blot profiles from the analysis of cell cultured isolates revealed a remarkable stability. The minisatellite and LA6 probes showed no differences over time whereas the Tpr1 probe showed some minor variation with the presence of an additional band in time (Fig. 20). The telomere probe revealed usually minor differences in the short fragments over time although marked time-related differences were found in the Mazabuka isolate (Fig. 22). Profiles of parent isolates and their clones did not differ when using the minisatellite probe, whereas small differences were found between the parent isolate and the clones of two isolates (*T. parva* Mazabuka and Muguga) when using the other probes (LA6, Tpr1 and telomere probes). This reflects the multi component composition of these stocks. The LA6 and Tpr1 probe showed differences (presence of one additional band) between the Mazabuka parent isolate and its clones (Figs.20 and 21), and the telomere probe profiles revealed interclonal and marked time-related differences (Figs. 22 and 23) in the Mazabuka isolate (presence of additional bands and change of profile over time). The Tpr1 profiles for *T. parva* Muguga showed some minor variation with the presence of an additional band in time (Fig. 20). All other parent stocks and their clones showed consistent profiles over time and among their progeny using the four probes.

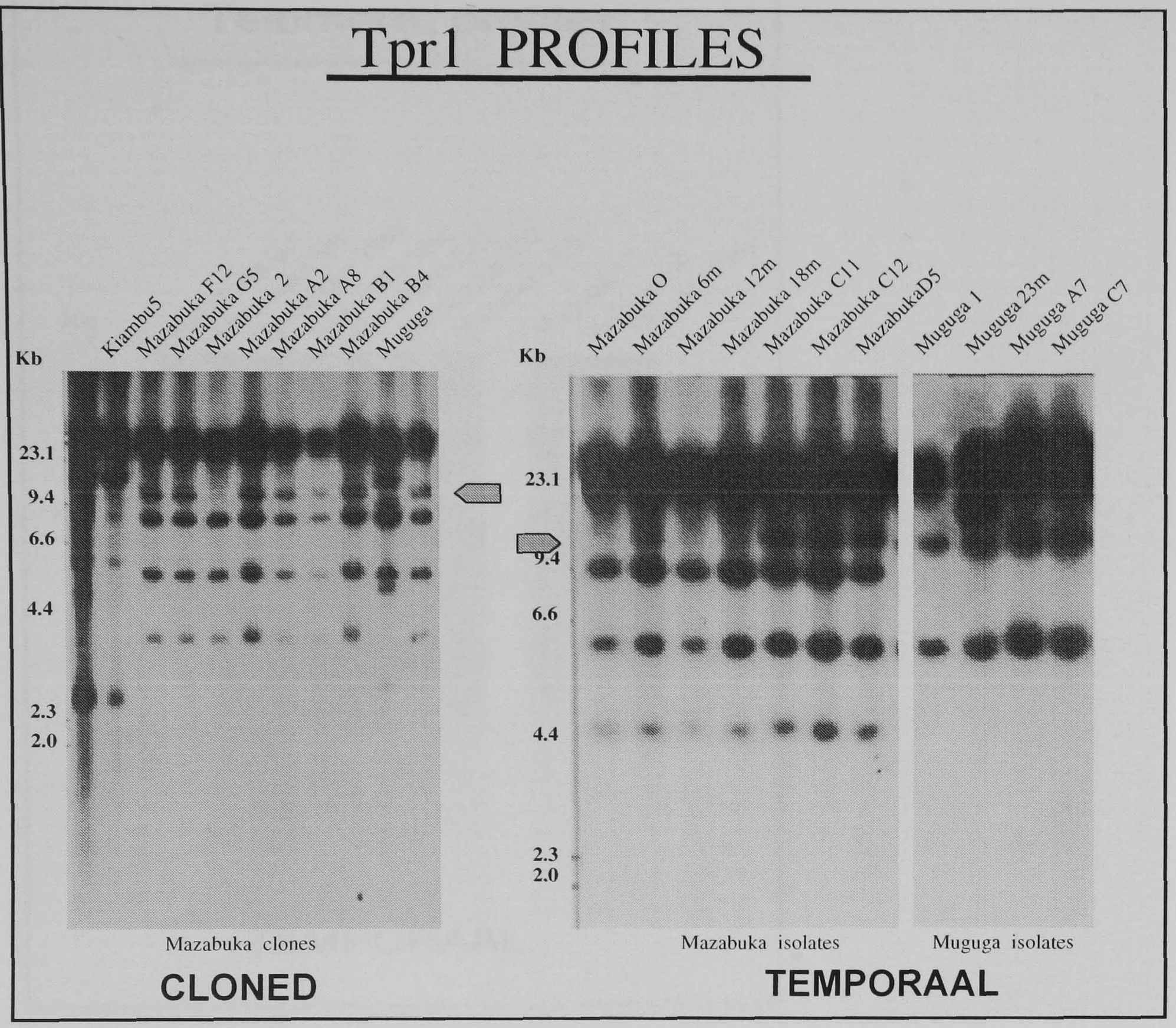


Fig. 20. Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with Tpr1. Patterns of *T. parva* clones and isolates over time. Arrows indicate absence of a band in some isolates.

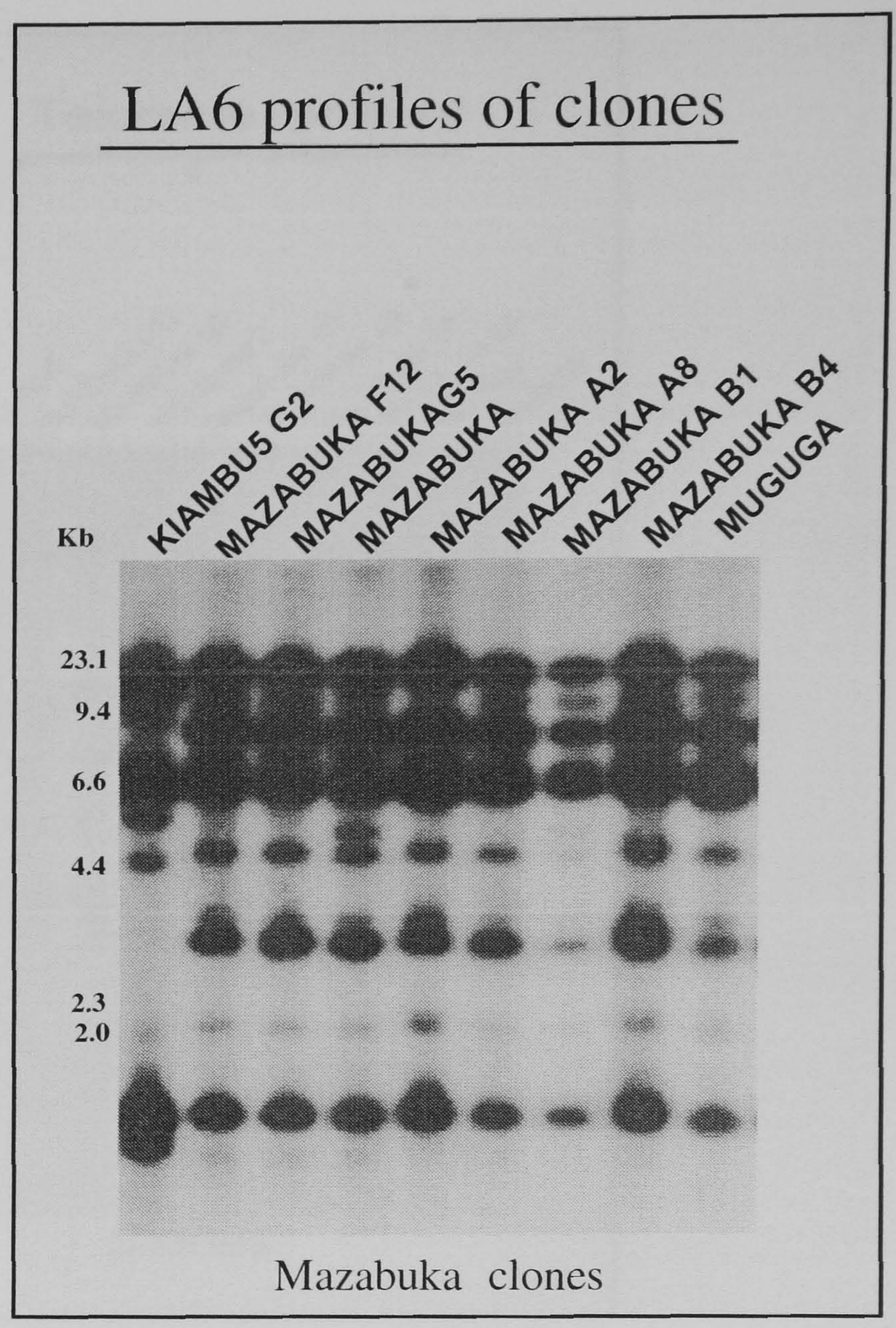


Fig. 21. Comparison of hybridisation patterns of *T. parva* Mazabuka DNA from cloned lymphoblastoid cells probed with LA6. *T. parva* Kiambu and Muguga are shown at the sides. A 5 Kb band present in the parent stock is absent in most clones (except in clone B1, although faint).

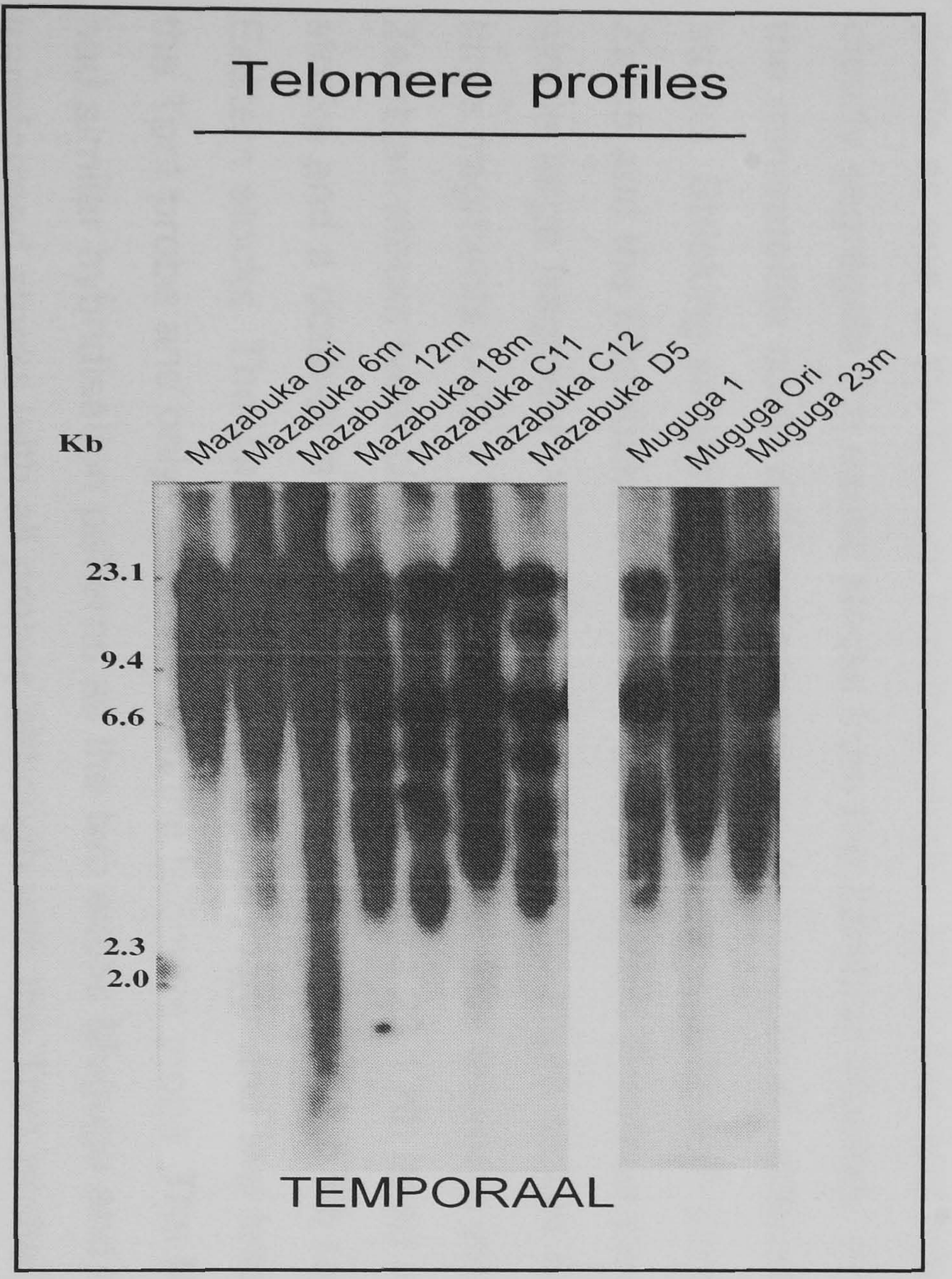


Fig.22. Comparison of hybridisation patterns over time of DNA from *T. parva* Mazabuka and Muguga cloned schizont-infected lymphoblastoid cells probed with the telomere probe. The Mazabuka original profile changes over time showing after 18 months a profile identical to its clones.

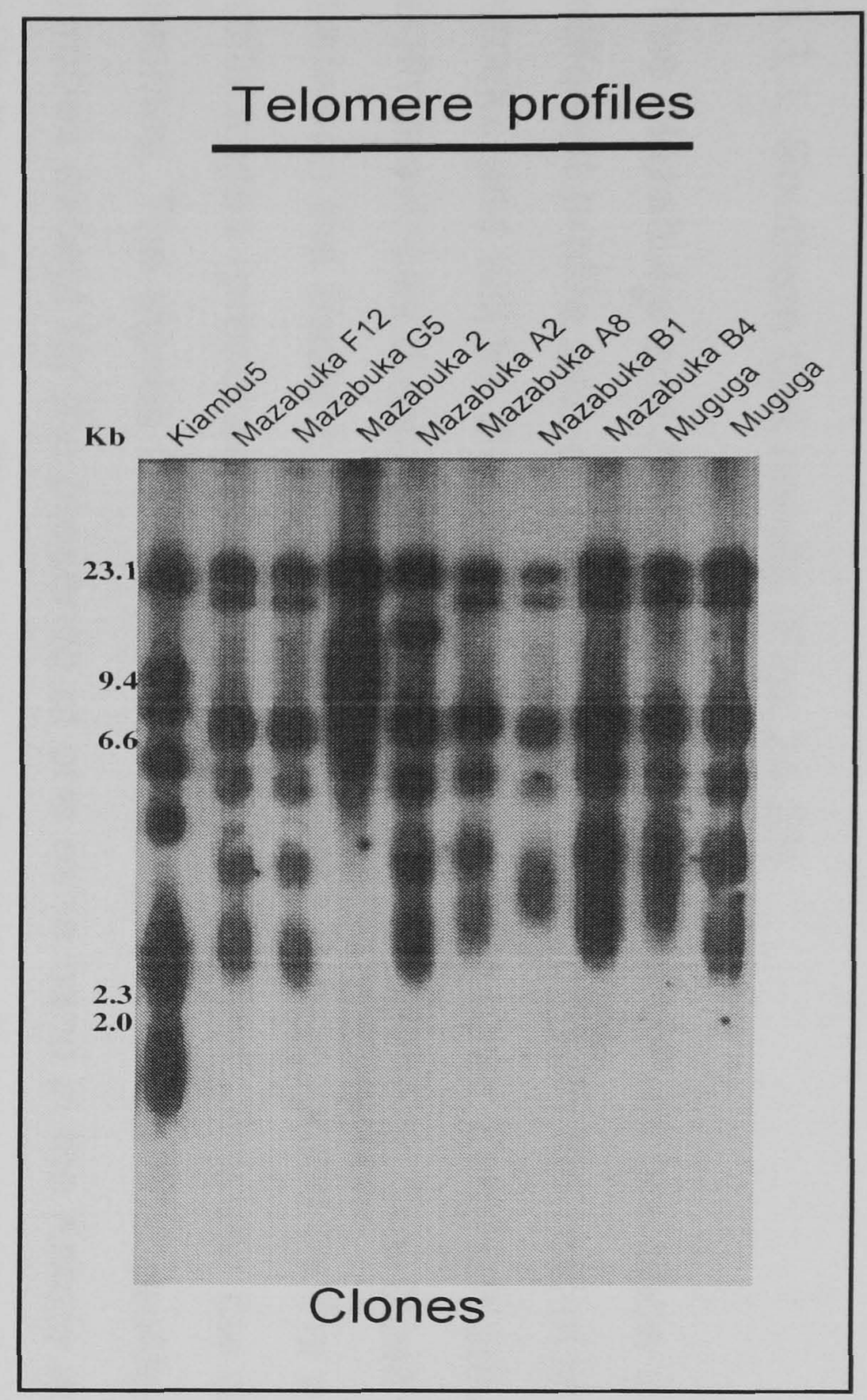


Fig.23. Comparison of hybridisation patterns of *T. parva* Mazabuka DNA from cloned lymphoblastoid cells probed with the telomere probe. *T. parva* Kiambu and Muguga are shown at the sides. Note the marked difference between the Mazabuka parent stock profile and the clones.

3.3. Characterisation of Theileria parva isolates

3.3.1. Southern blot results (Figs 24-28)

The Mazabuka and Zam5 stocks from Southern Province show a markedly different profile with all probes when compared with the rest of the Zambian stocks and will be discussed separately. The other Zambian stock profiles from both provinces display a high degree of homogeneity. The close relationship between the Eastern and Southern stocks is highlighted by the minor differences (presence/absence of one extra band) between the different probing profiles. The stocks from Eastern Province show identical profiles with all probes except for the presence of one extra band in the Katete and Langa stocks with the LA6 probe. The stocks from Southern Province show identical profiles with all probes except for the telomere probe hybridizing to one extra band in the Zam23.H2 clone. The Zambian profiles are closely related, contrasting with the pronounced differences found among the Kenyan profiles.

The LA6 probe was the only probe that could differentiate samples within the same geographical region. Two different LA6 profiles are found in stocks from Eastern Province although all Southern Province stocks showed the same profile as one of the Eastern Province stocks. All probes and markers could clearly segregate the exotic stocks from the Zambian isolates. All probes except the minisatellite could differentiate among geographically different Zambian stocks. Stricking similarities among the Zambian stocks with exception of the Zam5 and the Mazabuka stock were noted. Eastern Province stocks showed a single large fragment (>23.1 Kb) whereas the Southern stocks showed three large fragments (>23.1 Kb) with the Tpr1 probe. The telomere profiles of the Zambian stocks were clearly related with an extra 23.1 Kb band in the Southern stocks and a doublet in the small fragments between 1-2.5 Kb range in the Eastern stocks. The Zam5 stock showed four major and two lesser bands with the Tpr1 probe and only one band with the telomere probe. The Mazabuka stock had similar hybridisation patterns as the two exotic Muguga and the Serengeti-transformed stocks with all probes although with the Tpr1 probe minor

differences became apparent (Fig.24 and 25). Their profiles are however very distinct from the third exotic Kiambu stock and the Zambian profiles, including Zam5. The Muguga profile showed a band at 4.4 Kb also present in the Mazabuka profile but absent from the Serengeti-transformed profile. The Mazabuka stock showed an extra 3 Kb band but showed a unstable profile in the 6.6-9.4 Kb range with the occasional presence of one band (Fig24). The original isolates of Muguga and Serengeti-transformed showed a more complex polymorphism in this range than later passages. Most Southern blots of later passages of Muguga DNA stocks did not reveal a small 1.5 Kb fragment present in the Tpr1 probe profiles of the original Muguga and Serengeti-transformed isolates.

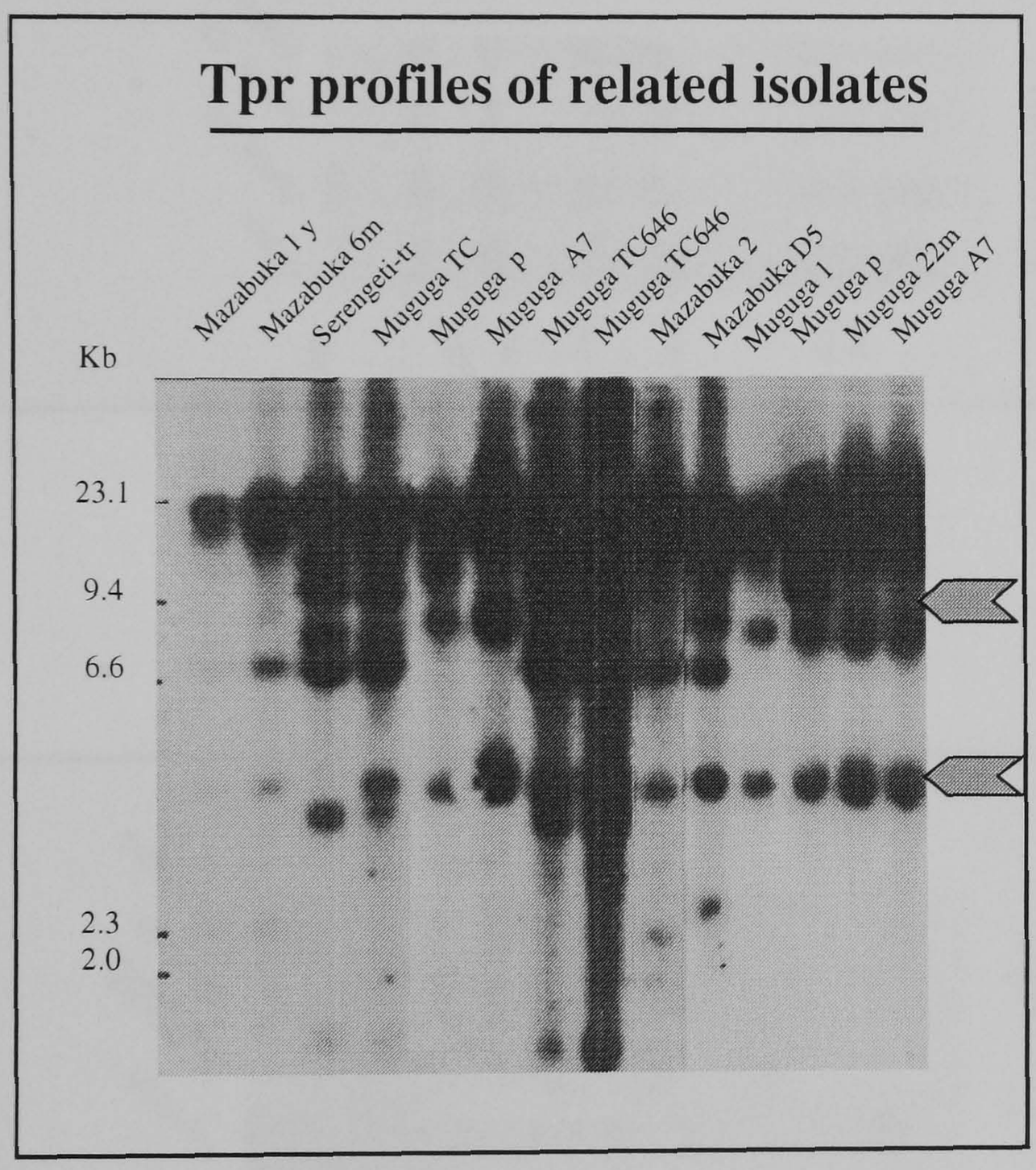


Fig. 24 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells of the closely related *T. parva* Muguga, Serengeti-transformed and Mazabuka isolates probed with Tpr1. Arrows indicate the position of polymorphic fragments.

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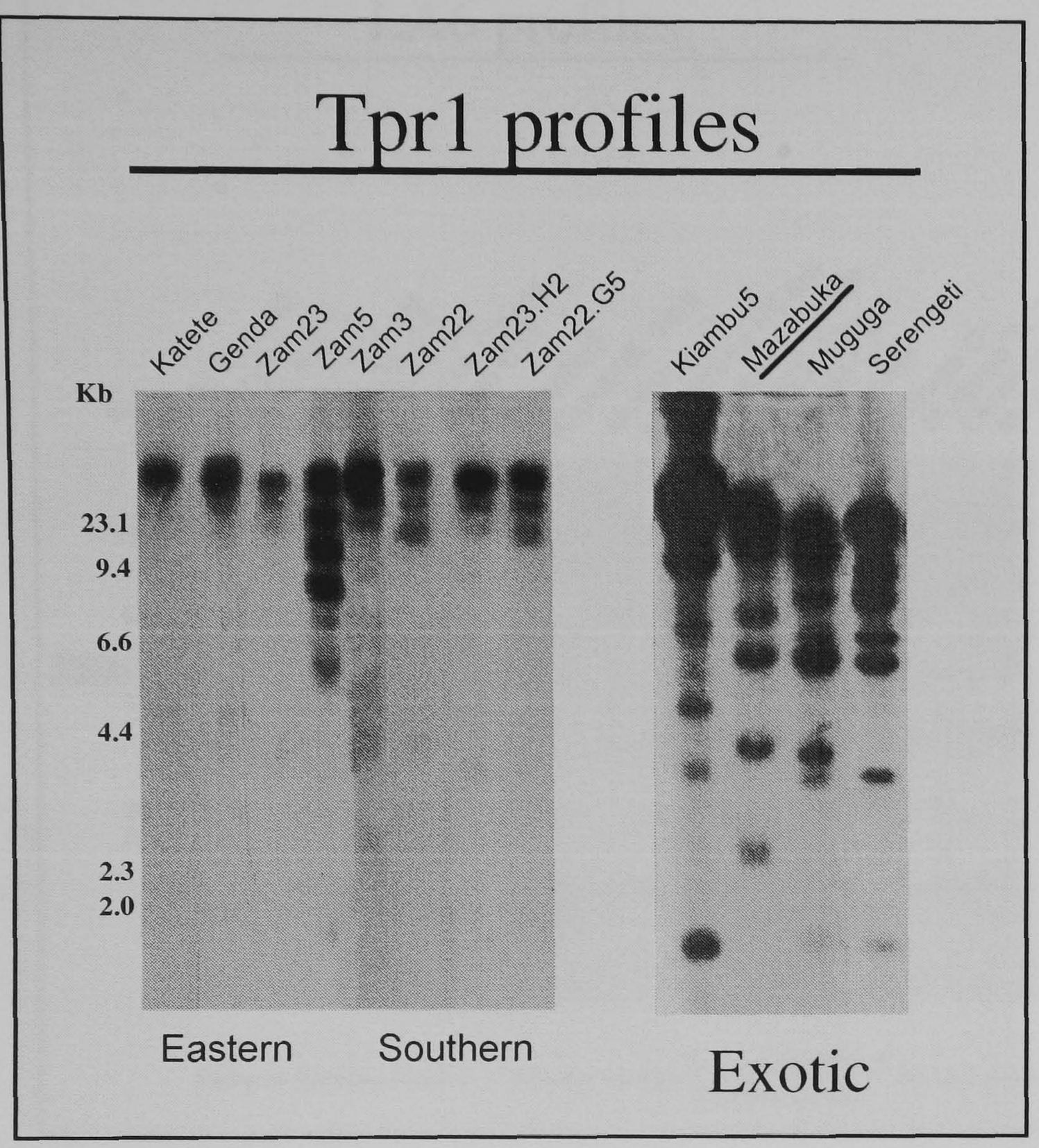

Fig.25 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with Tpr1. DNA's are from Eastern and Southern Zambian isolates and exotic isolates.

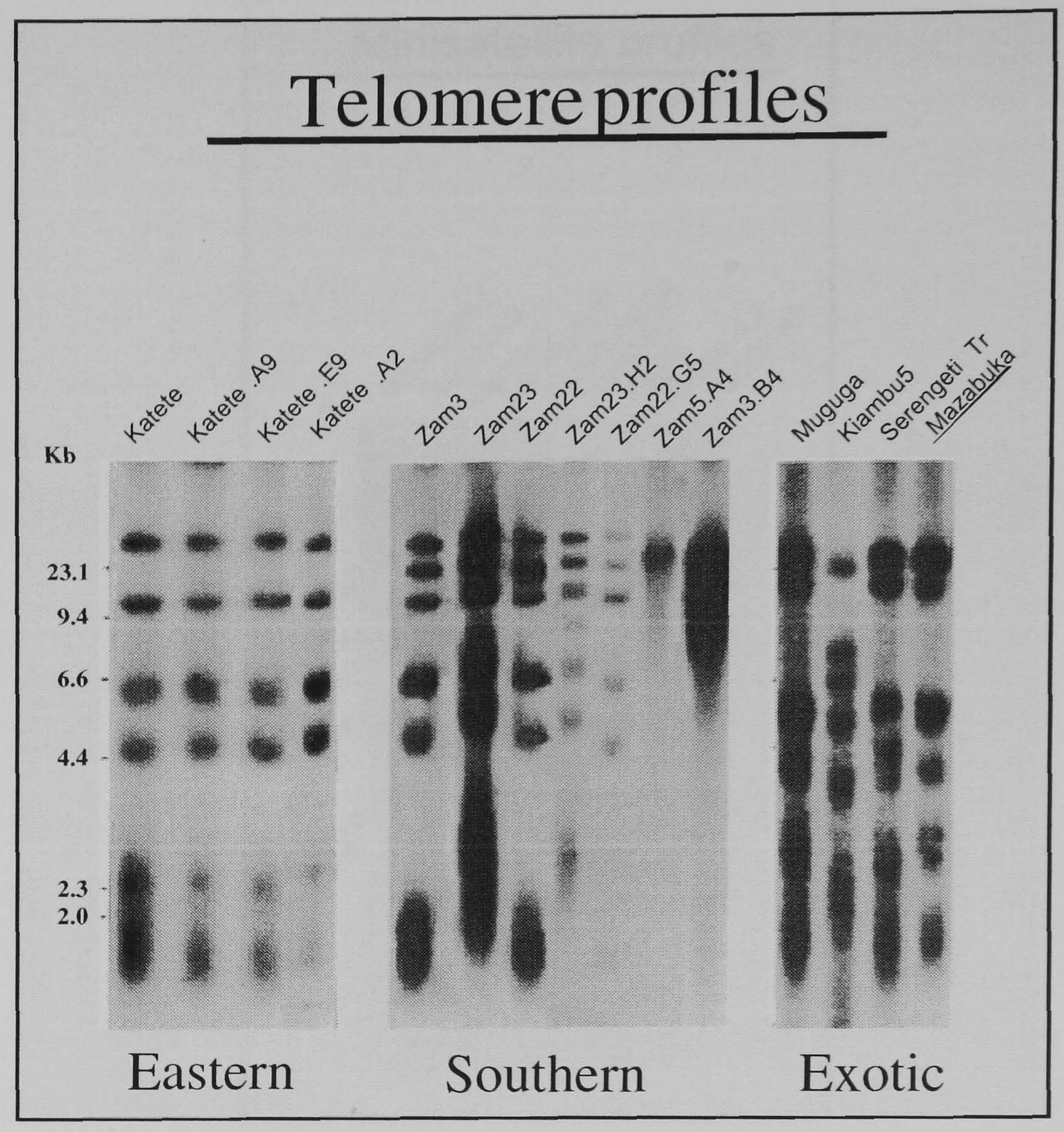

Fig.26 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with telomere. DNA's are from Eastern and Southern Zambian isolates and exotic isolates

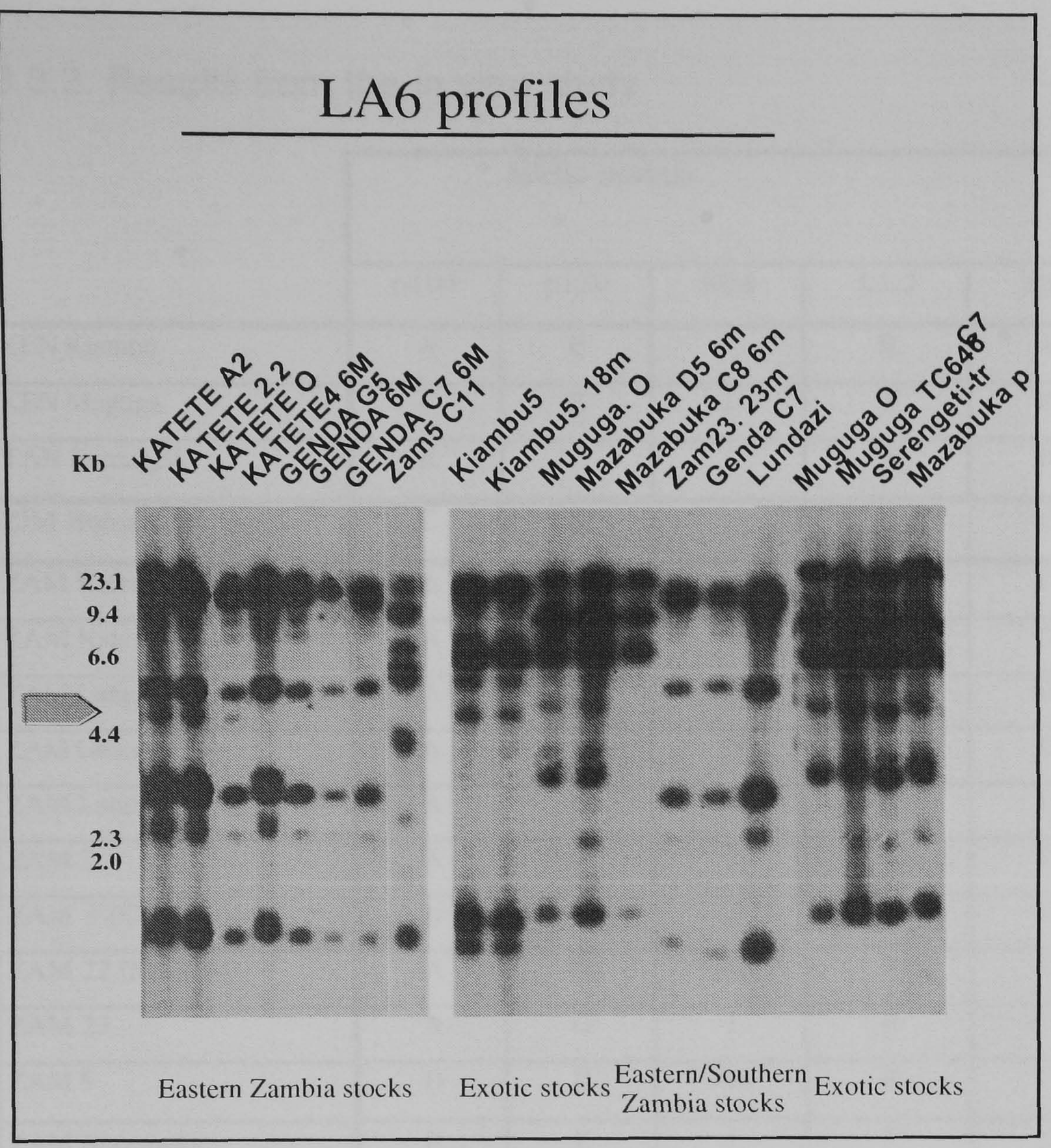

Fig.27. Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with LA6. DNA's are from Zambian and exotic stocks with the arrow pointing at the extra 5.5 kb fragment in the Katete stock.

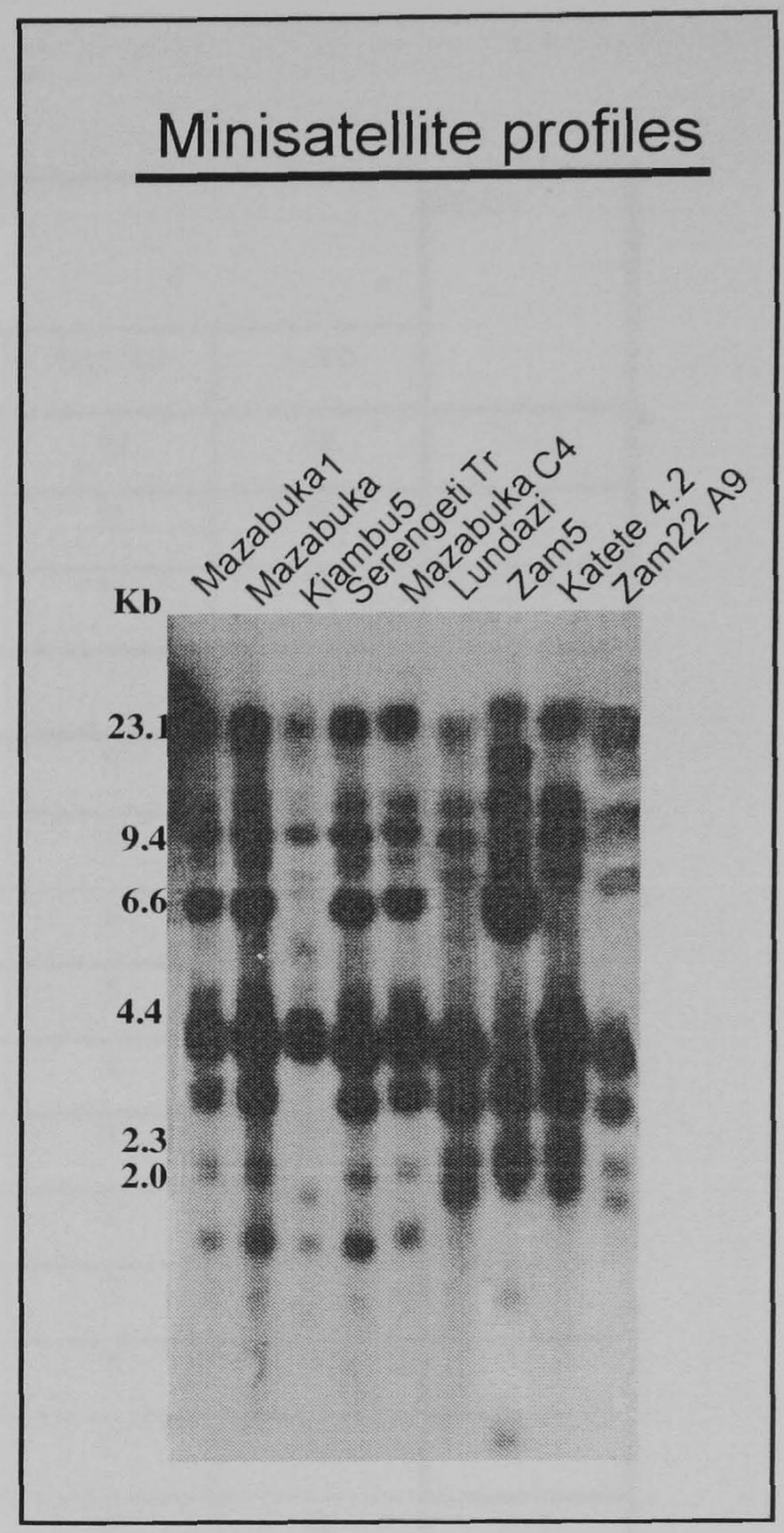

Fig.28 Comparison of hybridisation patterns of DNA from schizont-infected lymphoblastoid cells probed with Minisatellite. DNA's are from Eastern and Southern Zambian isolates and exotic isolates.

3.3.2. Results from the in vitro study

	Allelic markers					RFLP probes				Mabs
	p104	p150	PIM	L1,2	Tpr	Telo	Tpr1	Minisat	LA6	
KEN Kiambu	A	E	P	R	A	A	G	Q	M	7-15
KEN Muguga	B	E	I	T	B	B	H2	R	N	2-7
TAN Serengeti	B	E	I	T	B	B	H3	R	N	2-7
ZIM Boleni	C	F	U	S	C	C	I		O	0
ZAM Katete	A	G	L	R	D	D1	J1	S	P1	7
ZAM Katete4	A	G	O	R	D	D1	J1	S	P1	7
ZAM Langa	A	G	L	R	D	D1	J1	S	P1	7
ZAM Genda	A	G	O	R	D	D1	J1	S	P2	7
ZAM Lundazi	A	G	O	R	D	D1	J1	S	P2	7
ZAM 2	A	G	J	R	D	D3	J2	T	P2	7
ZAM 3 (Chitongo)	A	G	J	R	D	D3	J2	T	P2	7
ZAM 22 (Mandali)	A	G	J	R	D	D3	J2	T	P2	7
ZAM 23	A	G	J	R	D*	D3	J2	T	P2	7
ZAM 5	D	H	M	R	E	E	K	U	Q	7
ZAM Mazabuka	B	E	I	T	B	B	H1	R	N	2-7
No. OF ALLELES	**4**	4	7	**3**	**4**	6	8	5	6	

Table 10. The results from the different loci and probes are presented by attributing letters to distinct profiles and additional numbers to related profiles. MAbs results are presented by the mAbs numbers (ILRI numbering from Minami *et al.,* 1983).* atypical result of Tpr1 amplification of Zam23, not seen in its derived clones

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3.3.3. Single locus results (Figs. 29-42)

3.3.3.1. SSCP-based p32 analysis

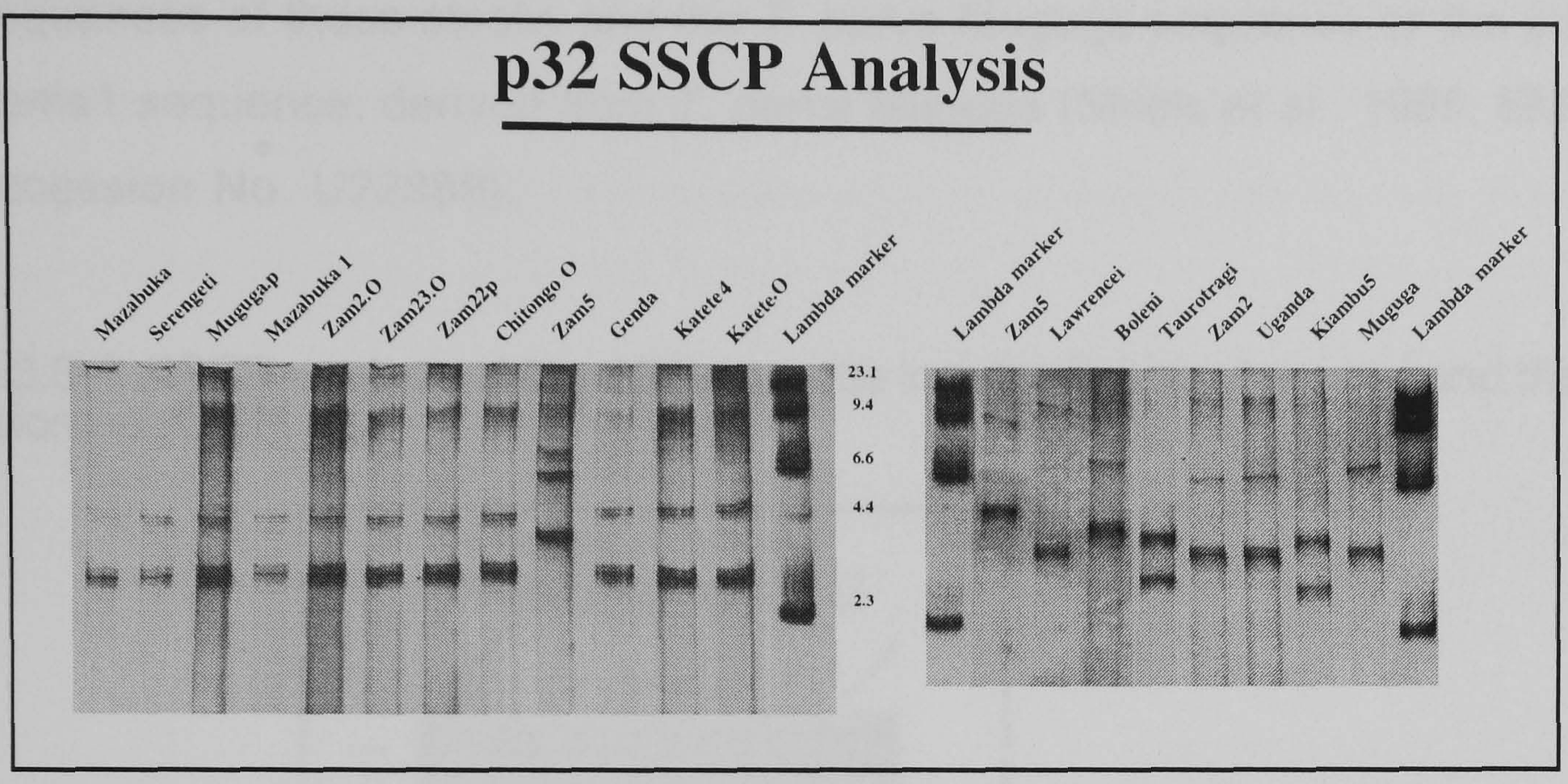

Fig.29. Comparison of SSCP profiles of PCR amplified p32 products of various *T. parva* isolates on a 10% PAGE gel.

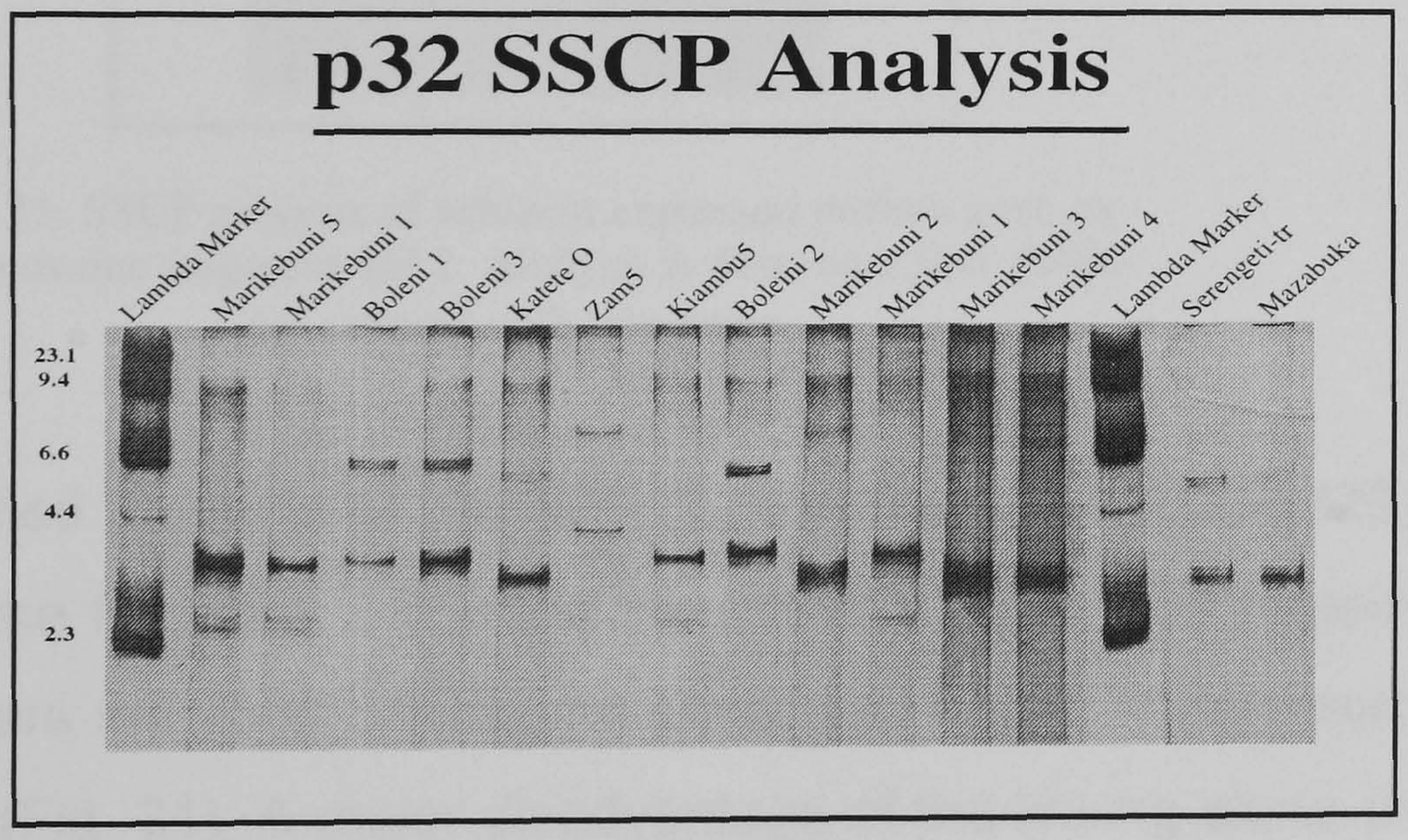

Fig.30. Comparison of SSCP profiles of PCR amplified p32 products of various *T. parva* isolates on a 10% PAGE gel.

SSCP profiles were obtained for the p32 locus and four different alleles were found. All Zambian stocks were identical to the Marikebuni 3 and 4, Uganda, Muguga, Serengeti-transformed and Mazabuka profiles. The Kiambu5, Marikebuni 1, 2 and 5 formed a second group with identical patterns, Lawrencei a third group, Boleni 1, 2 and 3 a fourth group whereas the Zam5 was different from the rest. These results suggest polymorphism of the p32 gene subdividing

the cattle-derived isolates in two groups and the buffalo-derived isolates in three groups.

PCR generated p32 products of *T. parva* Katete (Eastern Province) and Chitongo (Southern Province) were sequenced. No difference was found between the sequences of these stocks and the *T. parva* Muguga sequence or the partial Tpms1 sequence, derived from *T. parva* Muguga (Shiels *et al.*, 1995; EMBL accession No. U22889).

3.3.3.2. SSCP analysis of the ABC cassette loci, the linking clone loci and the telomeric ORF

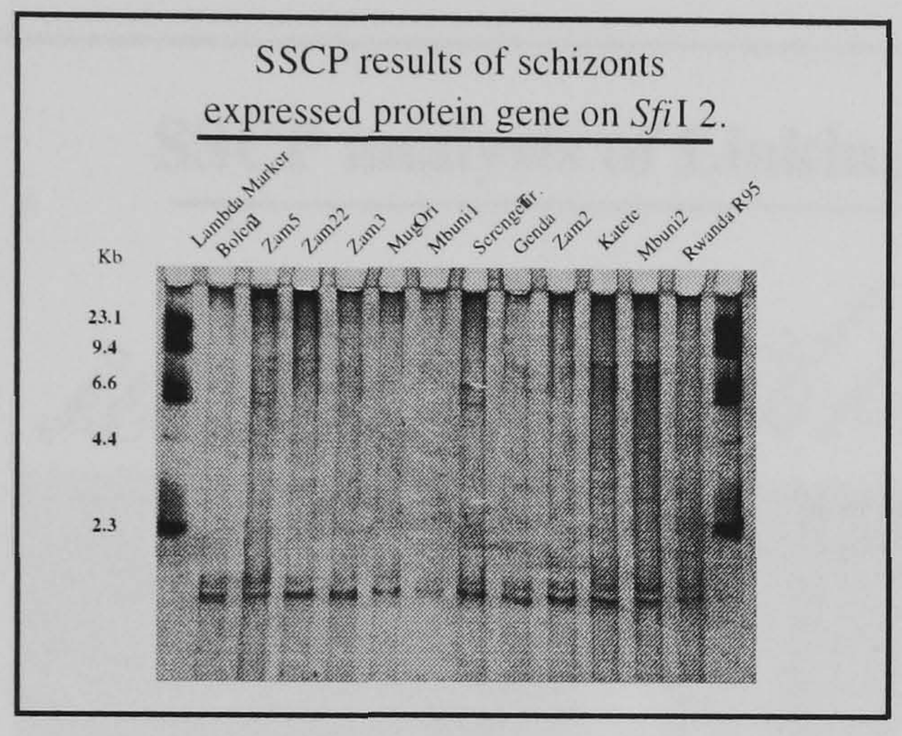

Fig. 31. SSCP analysis of schizont expressed protein gene on chromosome fragment *Sfi*I 2. Analysis is done on a 10% PAGE gel, colored with silverstain.

The amplified products of the ORF (ILRI primers nrs 3830/3829) on chromosome fragment *Sfi*I 1 and the schizont expressed protein gene on *Sfi*I 2 (ILRI primers nrs 756/757) did not show size or SSCP polymorphism among the 13 stocks (Fig. 31). A major disadvantage of the linking clone primers was the low yields in PCR amplification which could not be improved by varying annealing temperatures or Mg concentration.

Amplification using the linking clone primers gave single fragments of 250 bp length with L1-L2 and L3-L4 while a more complex group of fragments was amplified using the L5-L6 and L7-L8 primers. SSCP analysis of the L1-2 locus gave dimorphism between the Kenyan and the Zambian isolates including Zam5.

The results from the L3-4 locus revealed four different profiles. Isolates from the Eastern Province showed two different profiles with Genda differing from Katete

but identical to the Zam23 isolate. A third group was formed by the Muguga, Serengeti-transformed, Mazabuka and Kiambu isolates with identical profiles but different from the profile of Boleni and Zam5 isolates which formed a fourth group.

The amplification products of the L5-6 locus were very complex and resulted in complex SSCP profiles which could not be analysed. The SSCP analysis of the L7-8 locus gave a dimorphism separating the Zambian profiles, including Zam5 and Kiambu from the Muguga, Serengeti-transformed, Mazabuka and Boleni isolates.

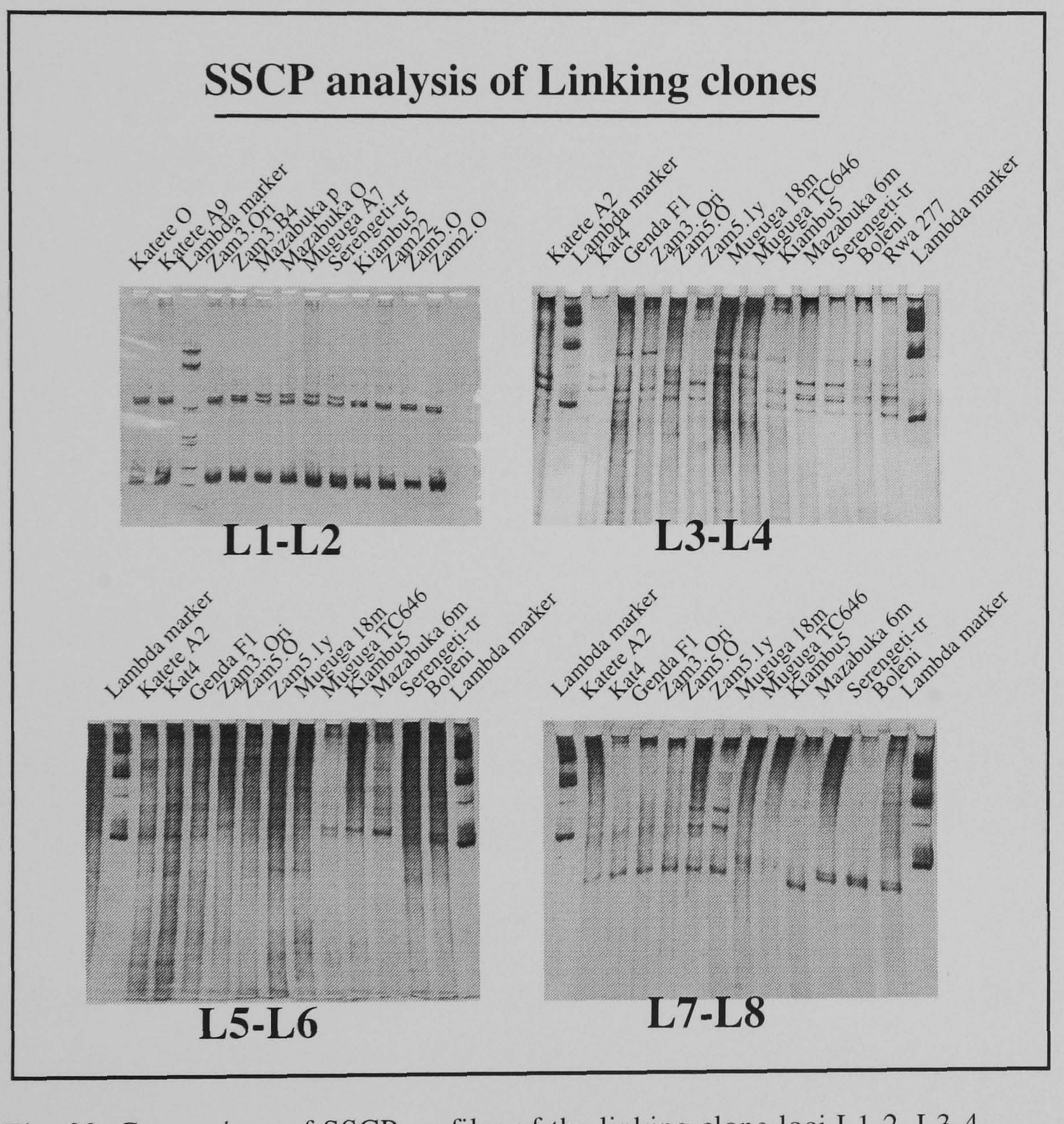

Fig. 32. Comparison of SSCP profiles of the linking clone loci L1-2, L3-4, L5-6 and L7-8 on a 10% PAGE gel, colored with silverstain.

The linking clone products obtained by using ILRI primer pairs L1-L2 and L7-L8 all showed a trimorphism that could distinguish between the Zambian stocks, Zam5 and the exotic stocks, including Mazabuka. Due to this low polymorphism and difficult amplification of the linking clone loci further development was not

considered during this study although the L3-L4 could be attempted at a later stage.

ILRI primers nrs 4989/4990 did not amplify the Zambian stocks.

3.3.6. RFLP-PCR based allelic marker results

The PIM locus results of different isolates are combined in Fig. 35 and 36. Amplification of the PIM gene locus from various isolates showed marked size differences among the PCR products and 13 different alleles were identified. All isolates showed a single genotype with all fragments adding up to the predicted size (Table 11). But cloning of amplified PIM products from two parent Zambian stocks revealed a size polymorphism among a minority of the clones. These isolates (Zam3 and Kat.O) consisted of a mixture of a larger allele of the size revealed after PCR amplification and a smaller allele only revealed after cloning.

PIM Predicted *Bcl*I restriction fragments										
Isolate	Length	Restriction fragments in bp.								
Katete/B2	909				323	288	225			73
Genda S/A8	685				324	288				73
Chitongo/B30	915				326		258		129	
Chitongo S/C1	677				324		258			95
Zam22/C22	939				324		258		129	
Zam23/B26	913				324		258		129	73
Marikebuni	745				348/324					73
Muguga	940	750							117	73
Boleni	618				326			214		78
Zam5/C14	988		411	387					114	76
Lawrencei	1020				326		243	195/181		75

Table 11. Predicted PIM fragments lengths after *Bcl*I restriction digestion of amplified products using semi-nested PCR. S qfter the isolate name denotes the present of a smaller sized PIM allele in corresponding isolate. The clone number from which the sequence was derived is given after the isolate name.

RFLP-PCR analysis of DNA of successive cattle-tick passages of the original Katete isolate showed the presence of a dominant PIM allele (1000 bp) in the original isolate but all subsequent passages from tick passage two (Kat2) onwards revealed a smaller PIM allele of identical size (700 bp) as found in the Genda, Langa and Lundazi field isolates from Eastern Province. The RFLP-PCR results using the PIM assay showed the presence of two different but related genotypes in Eastern Province, and two markedly different genotypes (Zam5 and the rest) among the Southern Province stocks (Fig. 33). The RFLP-PCR results of the Zambian isolates differed substantially from *T. parva* Mazabuka, Muguga and Serengeti stocks (Fig. 34). The latter had identical profiles but differed from

the Kiambu5 (Fig. 34), Boleni, Lawrencei, Uganda and Marikebuni clones 1, 3 and 5 (Fig. 36).

The PIM locus results of the exotic stocks and four recent field samples from Southern Province (f-samples) are combined in Fig. 35. The results for the different Marikebuni stocks reveal the presence of different alleles. The Marikebuni 1 profile is identical to the Marikebuni stock profile, predicted from the sequence data. *Theileria parva* Marikebuni 2 and 4 are identical to the Muguga profile whereas Marikebuni 3 and 5 show similar but new profiles. The Boleni isolates all have the same profile, resembling the *T. lawrencei* profile. All PIM genes with documented sequence data generate the profiles predicted from computer simulation using the *Bcl*I restriction enzyme, except for the buffalo 7014 DNA sample. The majority of field samples have a profile identical to the *T. parva* Mazabuka, Muguga and Serengeti-transformed genotypes (see under 3.3.8).

The results clearly demonstrate the high discriminatory power of the PIM locus in differentiating among isolates from the same area and even between components of the same stock.

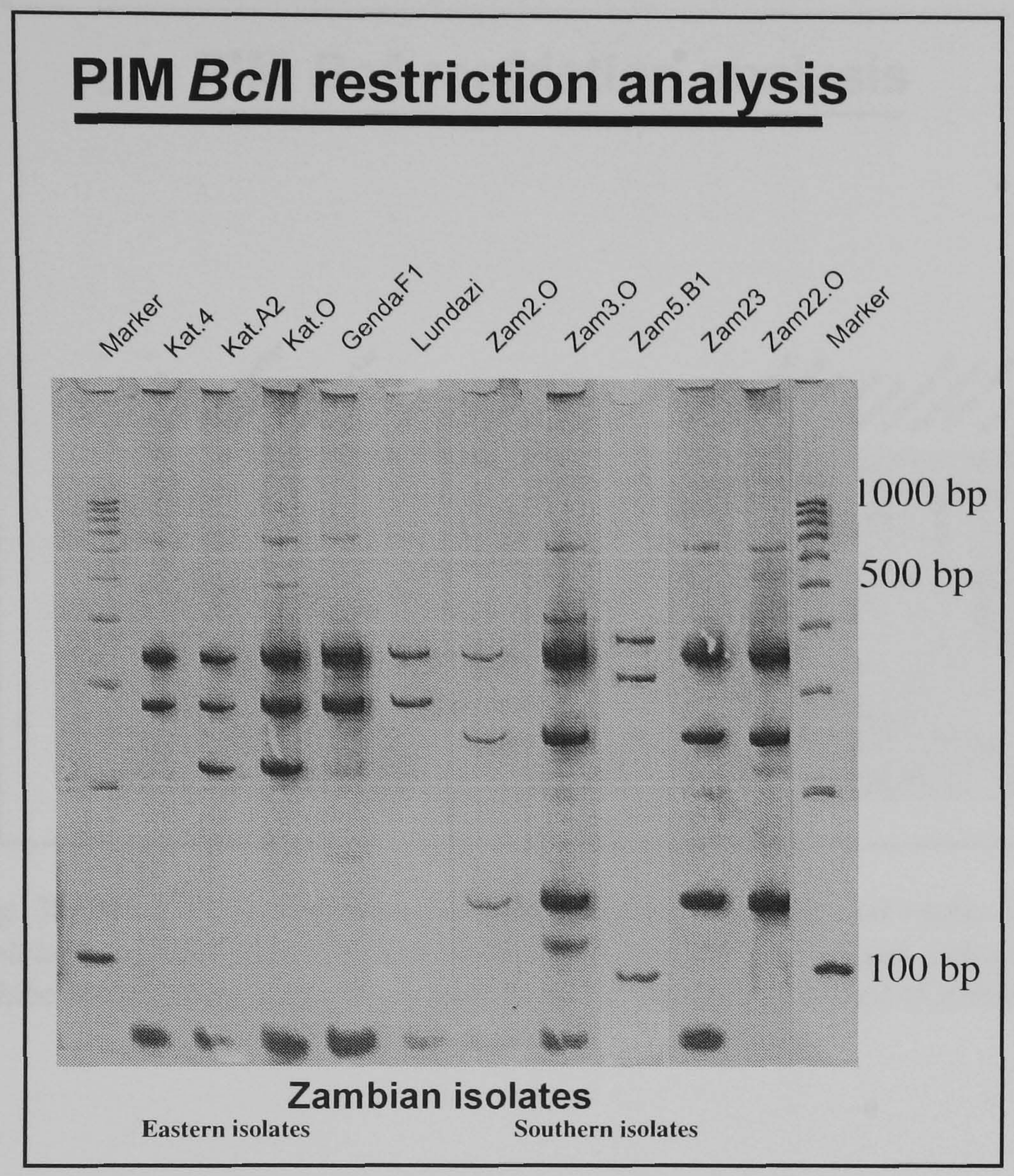

Fig.33. RFLP-PCR analysis of PIM locus from Zambian isolates using *Bcl*I digestion and fractionated on a on a 10% PAGE gel,stained with silver.

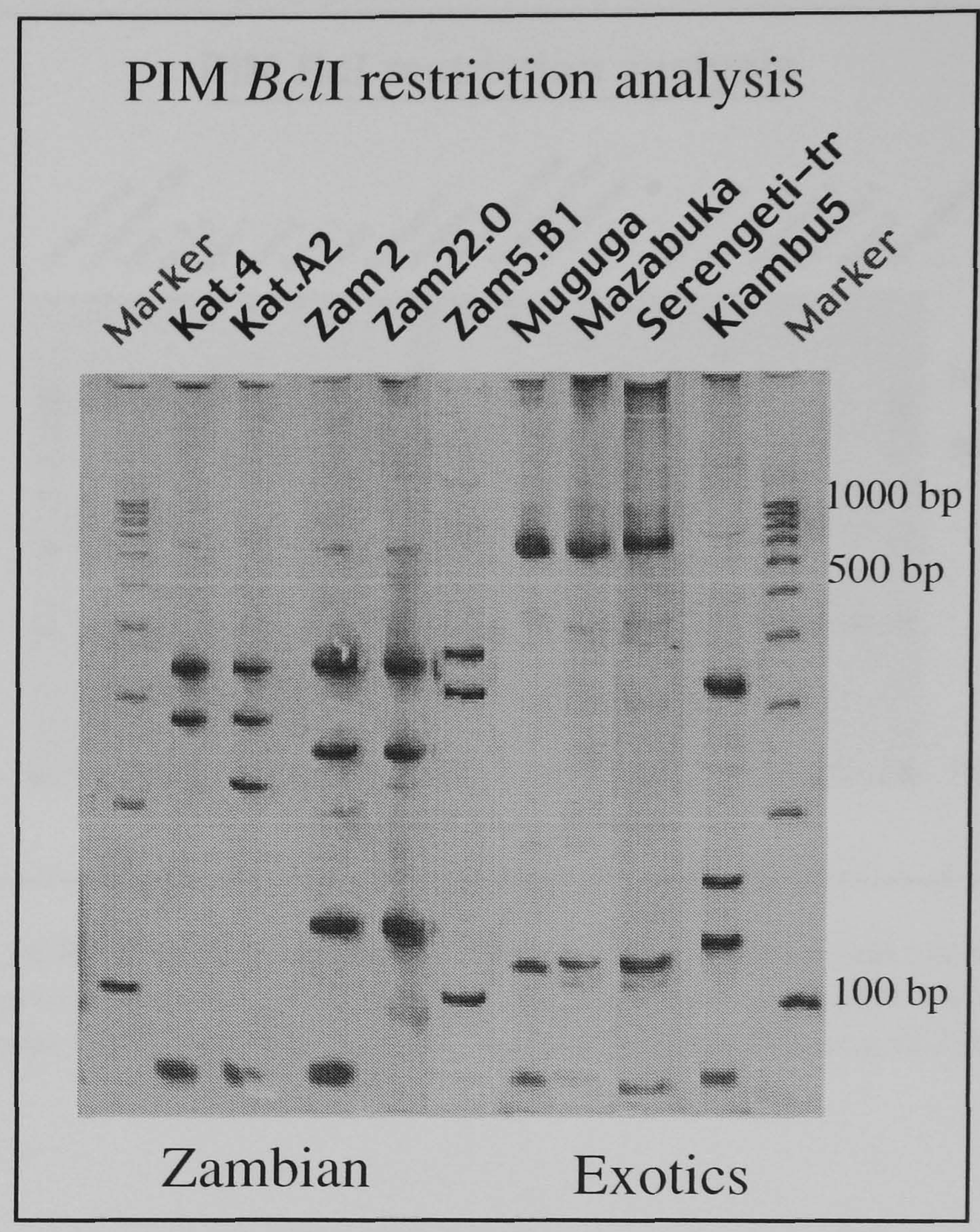

Fig. 34. RFLP-PCR analysis of PIM locus from Zambian and *T. parva* exotic *T. parva* isolates using *Bcl*I digestion and fractionated a 10% PAGE gel, stained with silver.

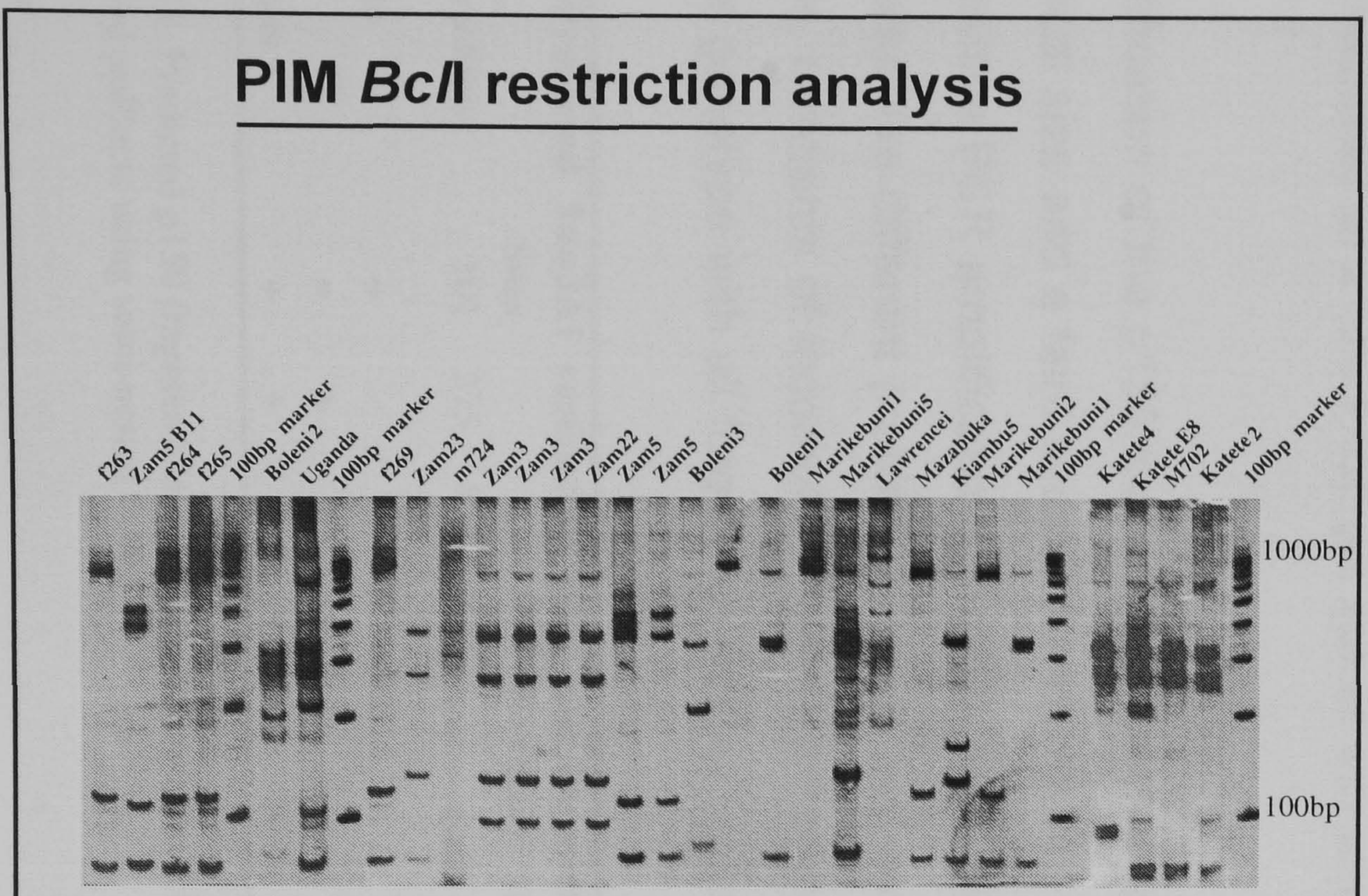

Fig. 35. RFLP-PCR comparison of PIM loci from Zambian and exotic *T. parva* isolates using *BclI* digestion and fractionated on a 10% PAGE gel, stained with silver. f-labelled numbers are field samples from the Southern Province of Zambia

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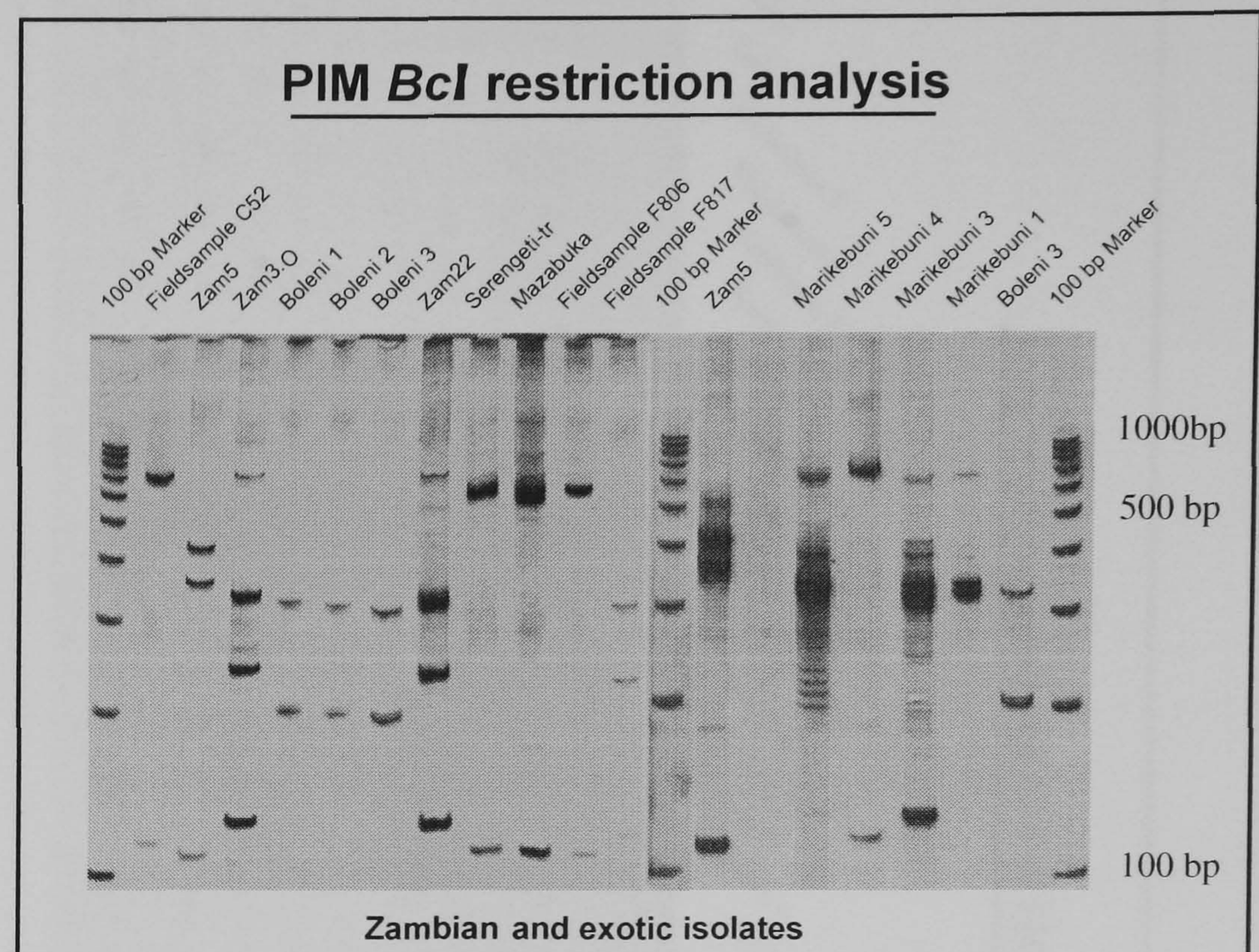

Fig. 36 RFLP-PCR comparison of PIM loci from Zambian and exotic *T. parva* isolates using *BclI* digestion and fractionated on a 10% PAGE gel Samples F and C are field samples from the Southern Province of Zambia.

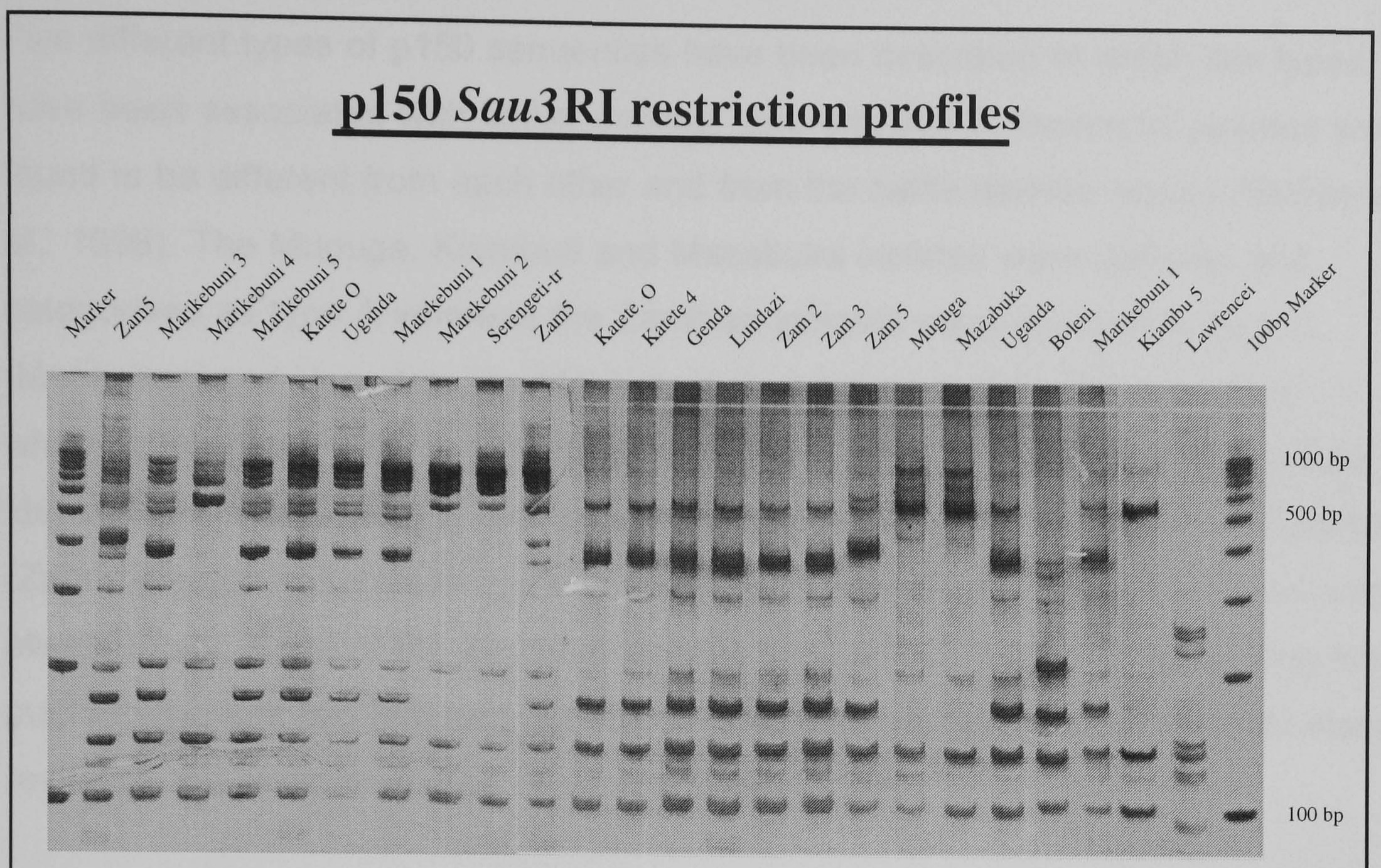

Fig.37. RFLP-PCR analysis using *Sau3R*I digestion of p150 loci from various *T. parva* isolates and fractionated on a 10% PAGE gel, stained with silver.

Amplification of the p150 locus of all isolates resulted in a single PCR product of identical size and a faint second band at 1400 bp detected on gel electrophoresis of all positive PCR amplifications for this locus. RFLP analysis of these products revealed five different profiles characterised by major fragments and some minor bands, remnants of incomplete digested fragments. Each profile corresponded to a single genotype with all fragments adding up to the predicted size (Table 12).

p150 Predicted *Sau*3AI restriction sites and fragments										
	Sites				Restriction fragments in bp.					
Length 940bp	101	275	657	717						
Muguga	+	-	-	+	616			299		101
Mbuni	+	+	+	+			382	299	174	101
Lawrencei	+	+	+	+		407		299	174	101

Table 12. Predicted p150 fragments lengths after *Sau*3AI restriction digestion of amplified products using semi-nested PCR.

Five different types of p150 sequences have been described of which two types have been associated with cattle isolates, whereas two *T. lawrencei* isolates were found to be different from each other and from the cattle-derived stocks (Skilton *et al.*, 1998). The Muguga, Kiambu5 and Mazabuka isolates were identical and categorised as type A whereas the Zambian isolates were identical to type C (Marikebuni and Uganda). The Marikebuni 2 and 4 were of the Muguga type whereas the Marikebuni 1, 3 and 5 were of the C type. The Boleni stocks all had identical profiles (type E) and *T. lawrencei* had a type B profile. A new p150 profile (Zam5 isolate) was revealed in this study and brings the number of different p150 alleles to six. None of the Zambian isolates showed a mixed genotype using this marker whereas two different genotypes were found among the Marikebuni clones revealing the complex nature of the Marikebuni stock

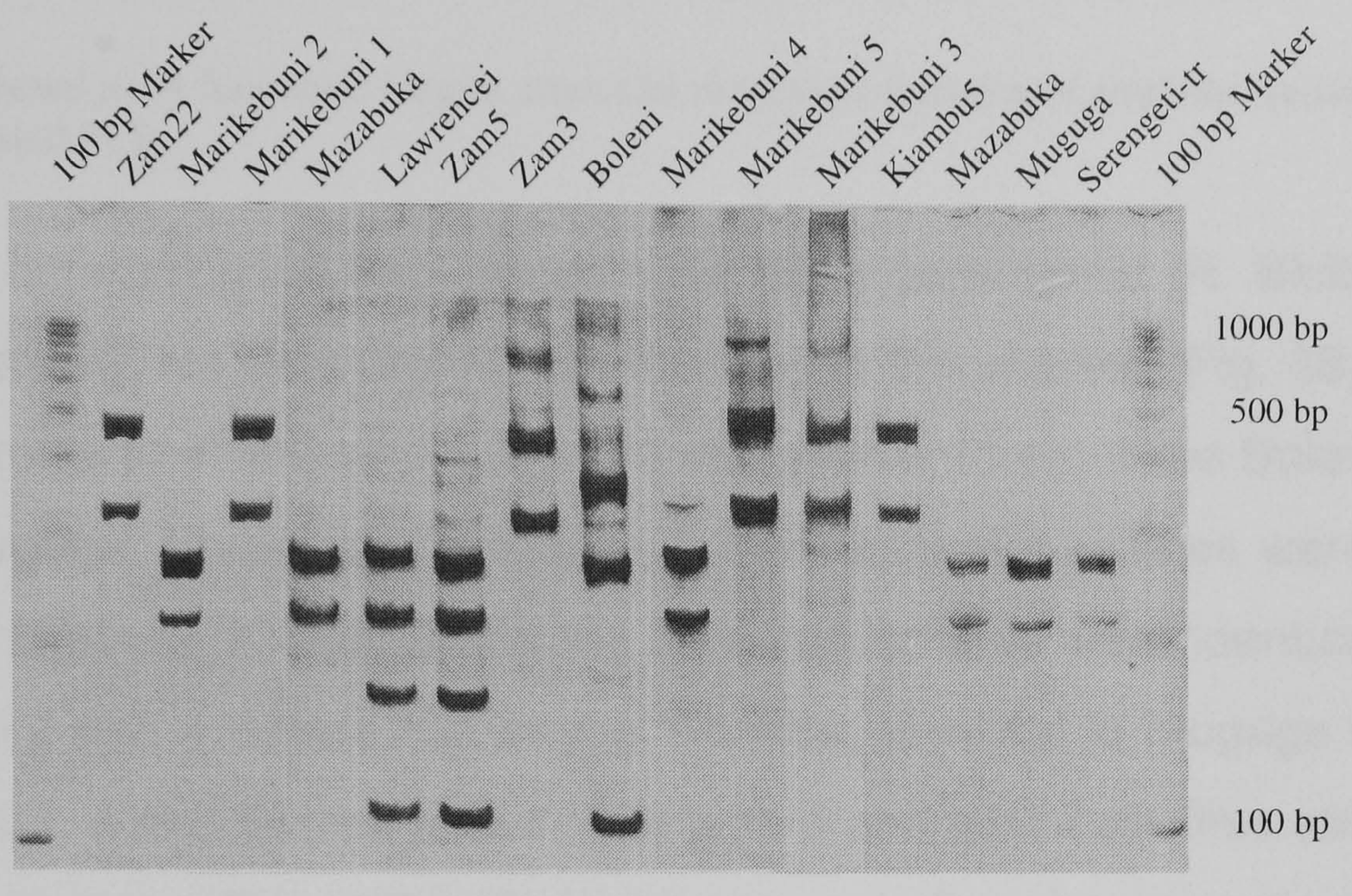

Fig.38. RFLP-PCR analysis using *Alu*I digestion of p104 loci from various exotic and Zambian *T. parva* isolates and fractionated on a 10% PAGE gel, stained with silver.

Amplification of the p104 locus of all isolates resulted in a single PCR product of identical size. RFLP analysis of these products revealed four different profiles characterised by major fragments and some minor bands, remnants of incomplete digested fragments. Each profile corresponded to a single genotype with all fragments adding up to the predicted size (Table 13). They reflect point mutations at sites 49, 439 and 607 and an *Alu*I restriction site at bp 330, present in all types (see Table 13).

p104 Predicted *Alu*I restriction sites and fragments									
	Sites				Restriction fragments in bp.				
	49	330	439	607					
Mbuni	-	+	-	-	498	330			168
Mug	+	+	-	+			281/277	221	
Boleni	+	+	+	-		389	281		
7014	+	+	+	+			281	221	

Table 13. Predicted p104 fragments lengths after *Alu*I restriction digestion of amplified products using semi-nested PCR.

In all, four different p104 sequences have been determined (R. Skilton, personal communication). No new profile was found with this marker (Fig. 38). Zam5 had an identical profile to *T. lawrencei* (type 4) and different from three Boleni isolates (type 3). The Muguga, Serengeti-transformed and Mazabuka isolates were identical (type 1) and the Uganda, Kiambu5 and the Zambian isolates were identical to type 2. The Marikebuni 2 and 4 isolate had a type 1 profile, identical to Muguga whereas Marikebuni 1, 3 and 5 were type 2. The type 1 and type 2 profiles are associated with cattle isolates. Two different genotypes were found among the Marikebuni clones confirming the complex nature of the Marikebuni stock. Most field samples from Southern Province (some shown as SP field in Fig. 38) show profiles identical to the *T. parva* Mazabuka, Muguga and Serengeti-transformed genotypes. Mixed RFLP profiles using the p104 assay were found in some Zambian field samples, revealing mixed infections between local and exotic genotypes.

18S locus results

All field samples were amplified using the 18S primers. The Msp1 restriction enzyme profiles provided clear distinctions between the various *Theileria* species (Fig. 39). The profiles of the different *Theileria* parasites were identical to the restriction sites predicted from known sequence data. No differences were found between the various *T. parva* groups including buffalo-derived isolates and *T. lawrencei* parasites. Most field samples yielded complex RFLP-profiles revealing multiple species infections. Southern Province field samples G111(Lane 12) and

G114 (Lane 14) are mixed infections by *T. mutans* and *T. buffeli* parasites whereas field samples G103 (Lane 2) and G109 (Lane 11) are examples of a *T. mutans* profile.

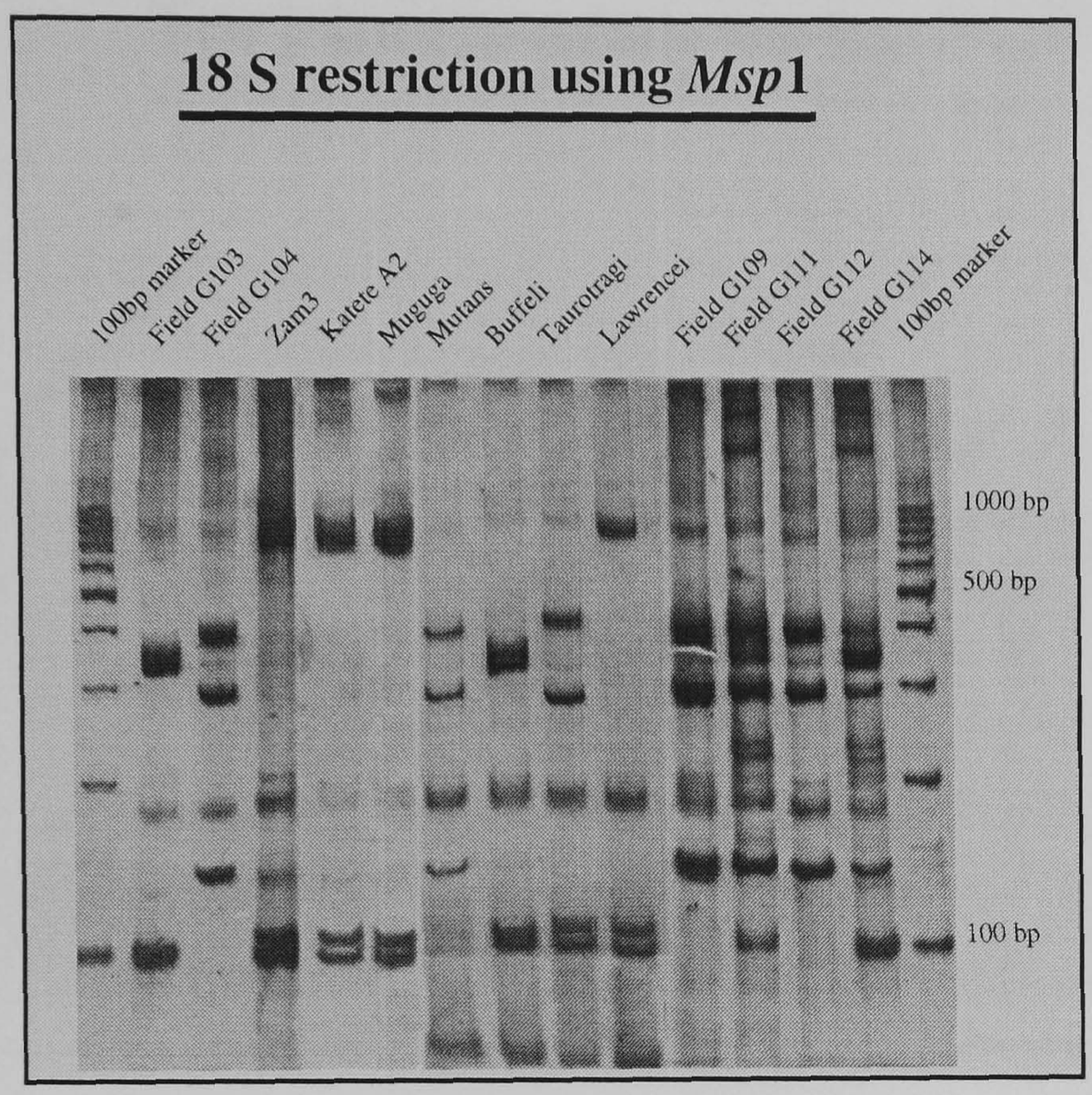

Fig. 39. RFLP-PCR analysis using Msp1 digestion of 18S loci from various *Theileria* isolates and fractionated on a 10% PAGE gel, stained with silver. Zam3, Katete A2 and Muguga are *T. parva* DNAs. Fieldsamples are from Southern Province of Zambia.

3.3.7. Size polymorphism analysis on gels

Tpr1 region

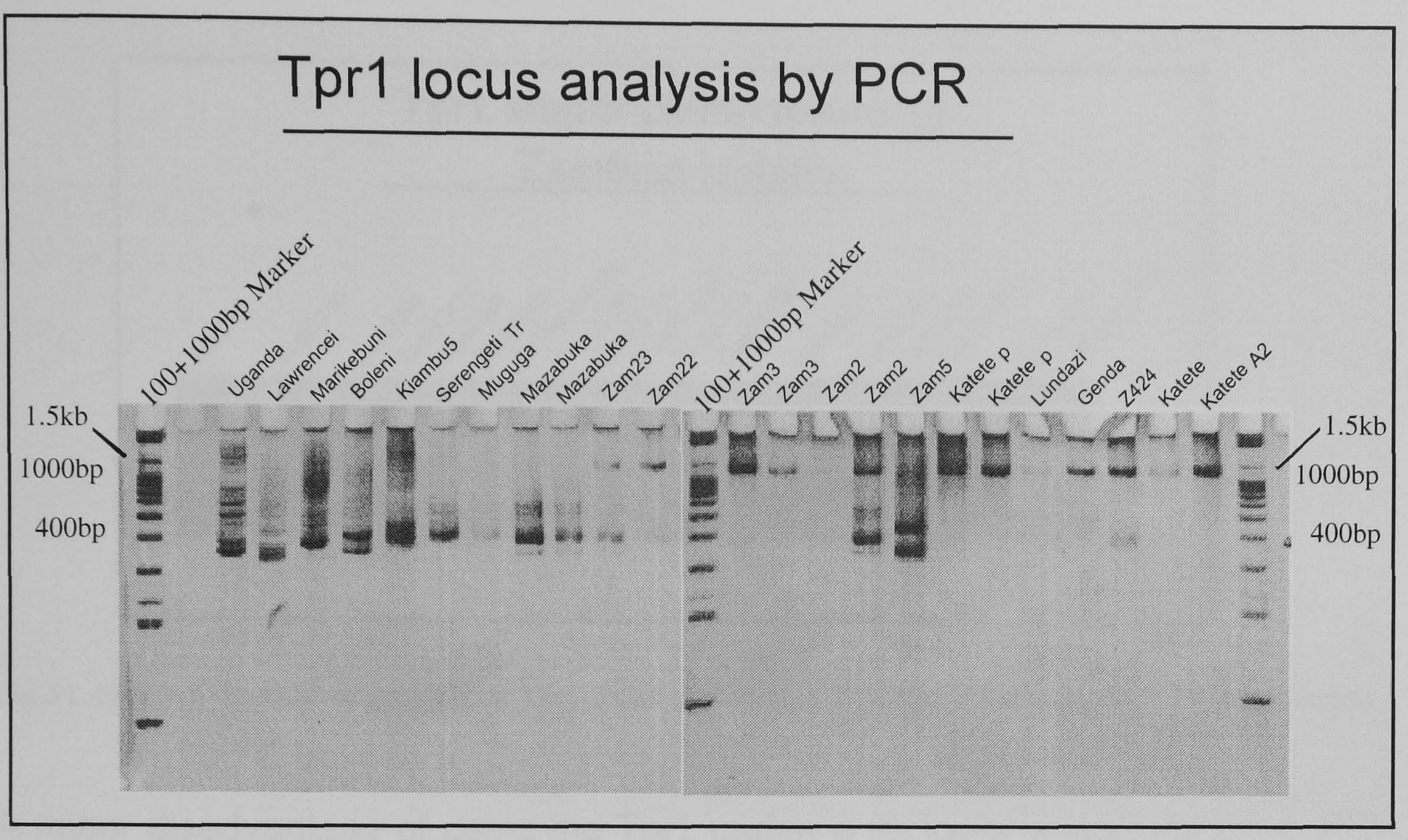

Fig.40. Size polymorphism analysis of Tpr1 locus of various *T. parva* isolates, using a 10% PAGE gel, stained with silver

The products of the Tpr1 locus revealed marked size differences which were best visualised on PAGE gels (Fig. 40). The Zambian stocks except Zam5 showed a large amplification product of 1400 bp. The Tpr1 products of all exotic stocks, including *T. lawrencei*, Boleni and Zam5, were around 400bp. Most exotic isolates showed weaker additional fragments resulting in a complex profile of fragments of different sizes. The Zambian isolates Langa and some of the Zam2 and Zam23 clones showed one or two weaker fragments in the 400bp range. The Zam23 parent stock revealed a profile differing from the latter ones, showing a strong amplification of 400 bp fragments and weak amplification of the 1400 bp fragment (Fig. 41).

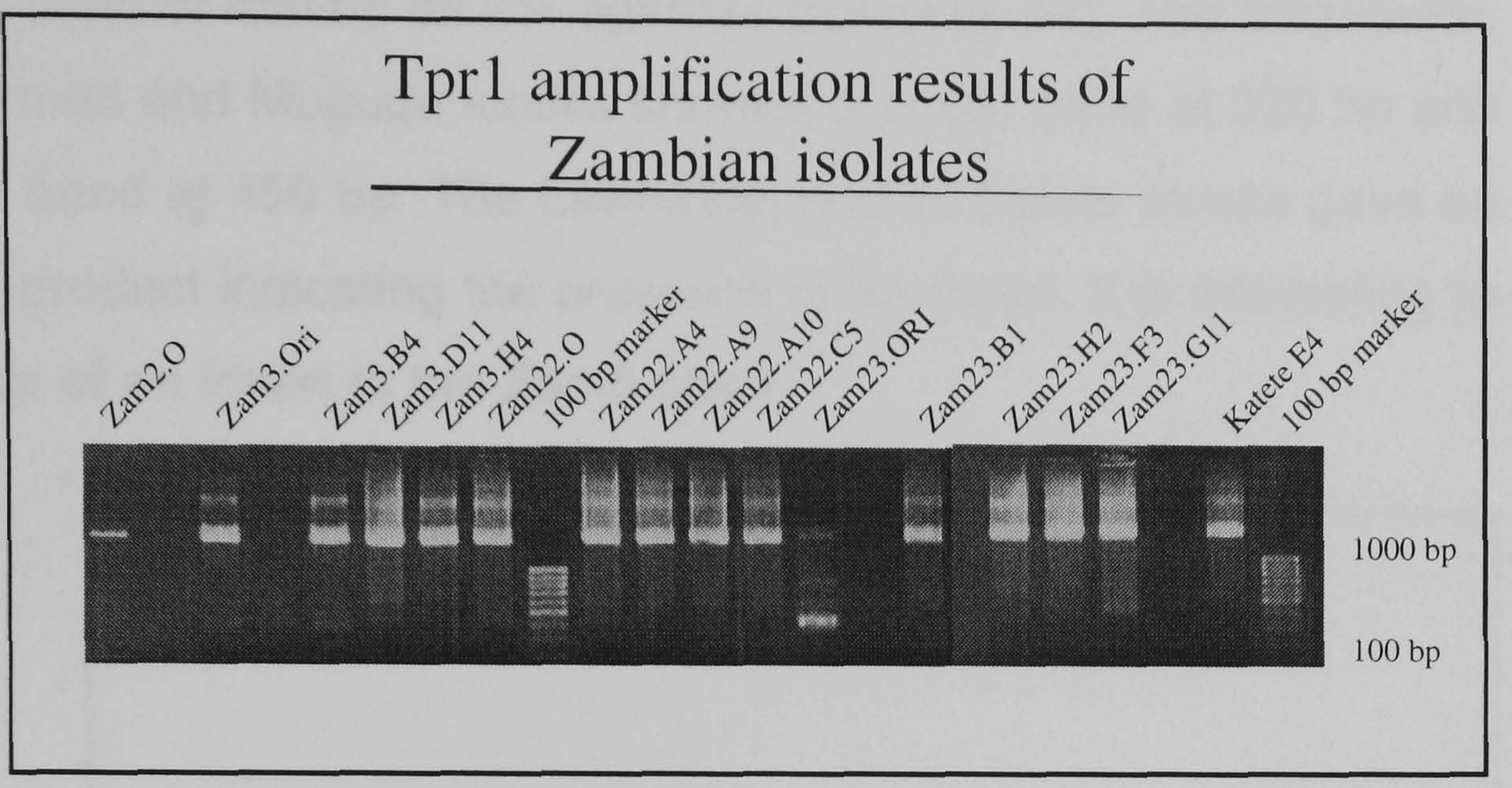

Fig.41. Size polymorphism analysis of Tpr1 locus of Zambian *T. parva* isolates, using a 2% agarose gel

A minor disadvantage of using the Tpr1 region is its lower specificity when PCR conditions are not followed strictly. When the annealing temperature is dropped slightly, Tpr1 regions of *T. taurotragi* and *T. mutans* are equally well amplified with a product of the same size as *T. parva* Muguga (390 bp).

p67 gene

All the Zambian stocks except Mazabuka showed a 320 bp product and a second weaker band of 450 bp on 2% agarose gels (Fig. 42). The Mazabuka, Serengeti-transformed and Muguga stocks showed a single band at 320 bp and a very faint second band at 450 bp. The Lawrencei and all Boleni stocks gave an approximately 400 bp product indicating the presence of an insert. It is interesting to note the absence of an insert in the Zam5 stock.

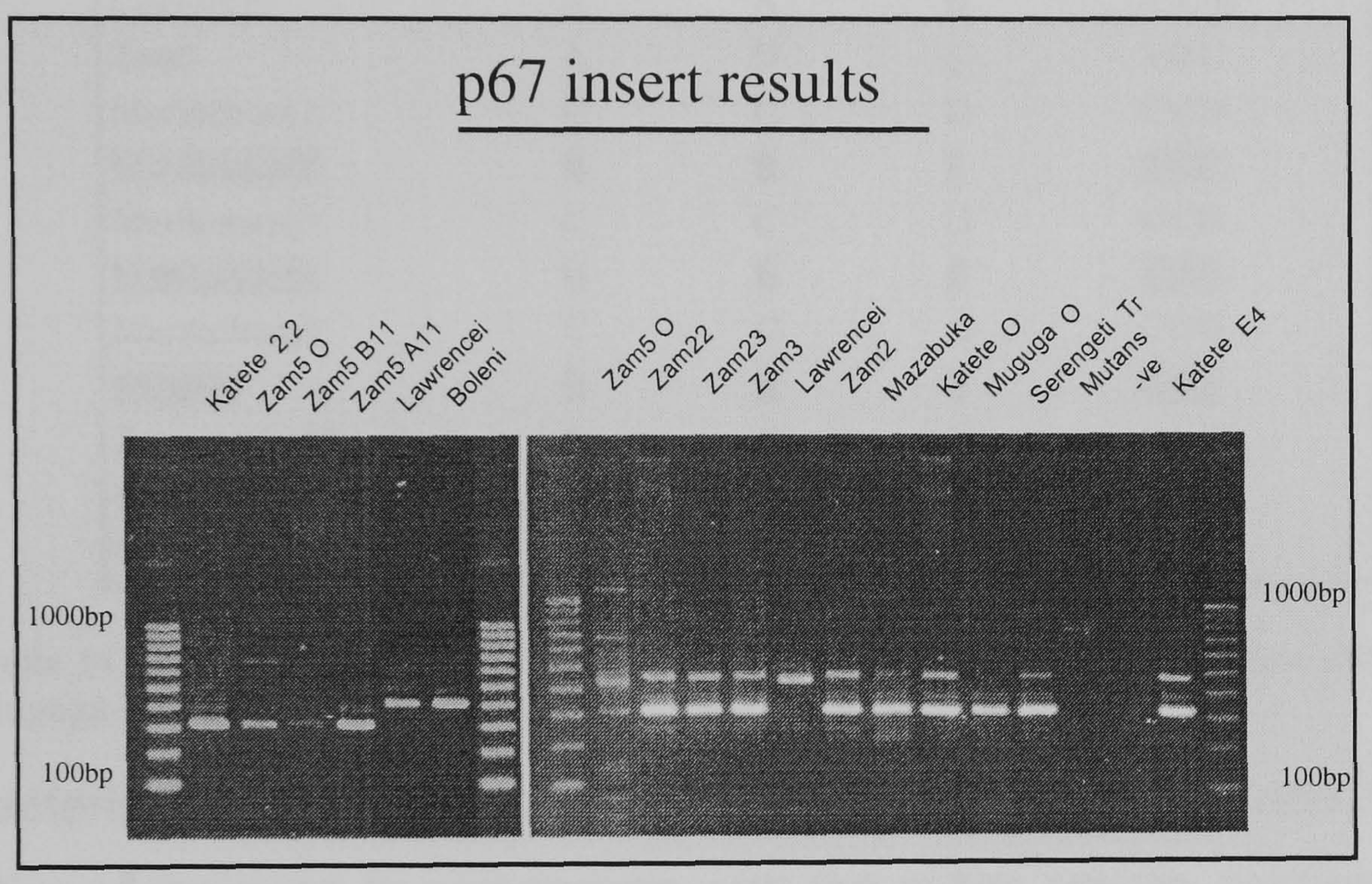

Fig.42. Size polymorphism analysis of the p67 locus of various *T. parva* isolates, using a 2% agarose gel.

Multilocus genotypes

A multilocus genotype can be derived by combining the single locus results and forms the basis for characterisation of parasite isolates (Table 14). It can be seen from the results presented in Table 14 that the Marikebuni stock is a complex isolate, shown to contain different genotypes only revealed after cloning.

locus stocks	**P104**	**P150**	**PIM**	**multilocus genotype**
Boleni	D	A	A	**DAA**
Lawrencei	A	A'	B	**AA'B**
Zam5	A	D	C	**ADC**
Marikebuni1	C	C	D	**CCD**
Marikebuni2	B	B	E	**BBE**
Marikebuni3	C	C	G	**CCG**
Marikebuni4	B	B	E	**BBE**
Marikebuni5	C	C	G	**CCG**
Muguga	B	B	E	**BBE**
Zams except Zam5	C	C	F	**CCF**
Kiambu5	C	B	H	**CBH**
Uganda	C	C	I	**CCI**

Table 14. RFLP-PCR results of multilocus characterisation. Marikebuni clones showing a Muguga genotype are highlighted.

The characterisation results (Table 10, page) using RFLP-PCR and Southern blot hybridisation techniques in comparison with the mAbs results confirmed the separation into different groups but reveal at the same time a much greater resolution. The minor differences in the hybridisation profiles of the Zambian stocks using the Tpr1 and LA6 probes are found to be more distinctly reflected in the RFLP patterns of the PIM locus. But this correlation between PIM and probe results is not found in Katete isolates of different passages giving different PIM patterns but identical probe profiles. The highest diversity was revealed by all Southern blot results using the different probes and the RFLP-PCR results of PIM but no correlation was found between PIM patterns and the probing profiles.

The unique position of the Zam5 isolate among the Zambian isolates is highlighted in both different hybridisation profiles and allelic marker patterns with the latter revealing new alleles for p150 and PIM.

3.3.8. Results from field surveys

A. Southern Province clinical cases

Chrushpen	District	Date	Nrs	+ve results					% +ve samples				
				Mug	South	New	Mixed	K5	Mug	South	New	Mixed	K5
Ngwezi	Mazabuka	May 96	7	7					1,0				
Hufwa	Monze	May 96	8	6			1	1	0,8			0,1	0,1
Ufwenuka	Monze	May 96	4	4					1,0				
Kazungula	Monze	May 96	8	8					1,0				
Hamavwa	Monze	May 96	11	3					0,3				
Nkonkola	Mazabuka	May 96	5	5					1,0				
NegaNega	Mazabuka	May 96	10	10					1,0				
Nadezwe	Mazabuka	May 96	4	4					1,0				
Sianjalika	Mazabuka	May 96	10	9	1				0,9	0,1			
Ngwezi	Mazabuka	Dec 96	1	0					0,0				
Hufwa	Monze	May 97	18	12					0,7				
Ngwezi	Mazabuka	May 97	2	2					1,0				
Ufwenuka	Monze	May 97	6	6					1,0				
Hufwa	Monze	May 97	52	33	1		1		0,6	0,02		0,02	
Total			**146**	**109**	**2**	**0**	**2**	**1**	**0,75**	**0,01**	**0,00**	**0,01**	**0,01**

Table 15. Details of PCR amplification from bovine samples from clinical cases from Southern Province, collected during 1996. Mug = Muguga profile; South = Southern Zambian profile; mixed = mixed Muguga and Kiambu5 and K5 = Kiambu5

B. Southern Province random samples

Chrushpen	District	Date	Nrs	+ve results					% +ve samples				
				Mug	South	New	Mixed	K5	Mug	South	New	Mixed	K5
Muzoka	Choma	Dec 96	9	0					0,0	0,0	0,0	0,0	0,0
Nega Nega	Mazabuka	Febr 97	3	0					0,0	0,0	0,0	0,0	0,0
Kanchomba	Mazabuka	Febr 97	3	3					1,0	0,0	0,0	0,0	0,0
Mbabala	Choma	Febr 97	6	2					0,3	0,0	0,0	0,0	0,0
Gamela	Choma	Febr 97	10	5					0,5	0,0	0,0	0,0	0,0
Batoka	Choma	Febr 97	12	2					0,2	0,0	0,0	0,0	0,0
Lubombo	Choma	Febr 97	6	0					0,0	0,0	0,0	0,0	0,0
Macha	Choma	Febr 97	4	0					0,0	0,0	0,0	0,0	0,0
Sikatumba	Monze	Febr 97	13	0					0,0	0,0	0,0	0,0	0,0
Ngwezi/Namucende	Mazabuka	Febr 97	13	1					0,1	0,0	0,0	0,0	0,0
Ufwenuka	Monze	Febr 97	1	0					0,0	0,0	0,0	0,0	0,0
Maamba	Choma	July 97	24	0					0,0	0,0	0,0	0,0	0,0
Bambwe	Choma	July 97	20	5					0,3	0,0	0,0	0,0	0,0
NamwalaC	Namwala	July 97	26	1					0,0	0,0	0,0	0,0	0,0
Chitongo	Choma	July 97	15	0					0,0	0,0	0,0	0,0	0,0
Total			**165**	**19**	**0**	**0**	**0**	**0**	**0,12**	**0**	**0,**	**0**	**0**

Table 16. Details of PCR amplification from bovine samples from suspected ECF-recovered animals from Southern Province, collected during 1996/97

From the 311 animals sampled in the Southern Province, 133 (43%) were found positive for *T. parva* parasites on PCR amplification using the PIM, p104 and p150 assays. All positive field isolates (N=133) were characterised and 128 animals found positive for parasites showing a Muguga/Serengeti multi-locus genotype. Two of these were mixtures of Muguga/Serengeti and Kiambu5 as revealed by the complex profile shown with the PIM assay, and one isolate showed a Kiambu5 profile (Fig. 44). Only two samples were showing a local Zam3 profile. In rare cases, PCR amplification of field samples resulted in the presence on a 2% agar gel of a weak fragment of atypical size. The geographical distribution of the positive samples in the Southern Province of Zambia is shown in Fig.34.

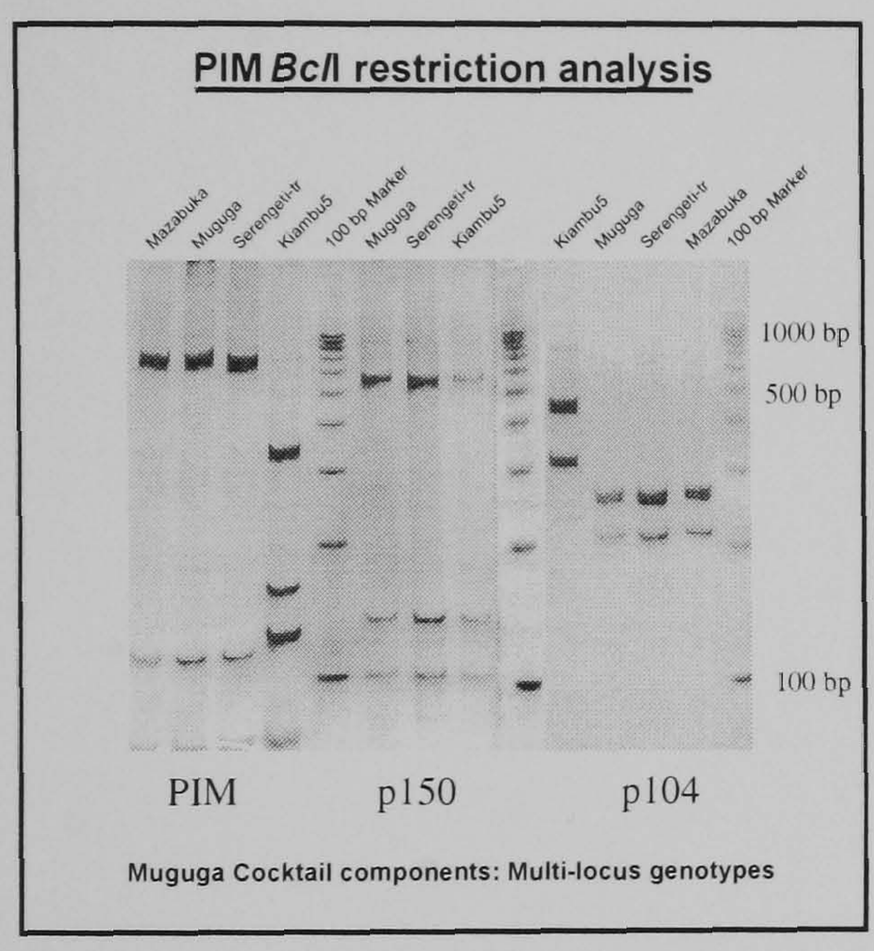

Fig. 43. Comparison of RFLP-PCR results of three loci of the different components of the cocktail vaccin

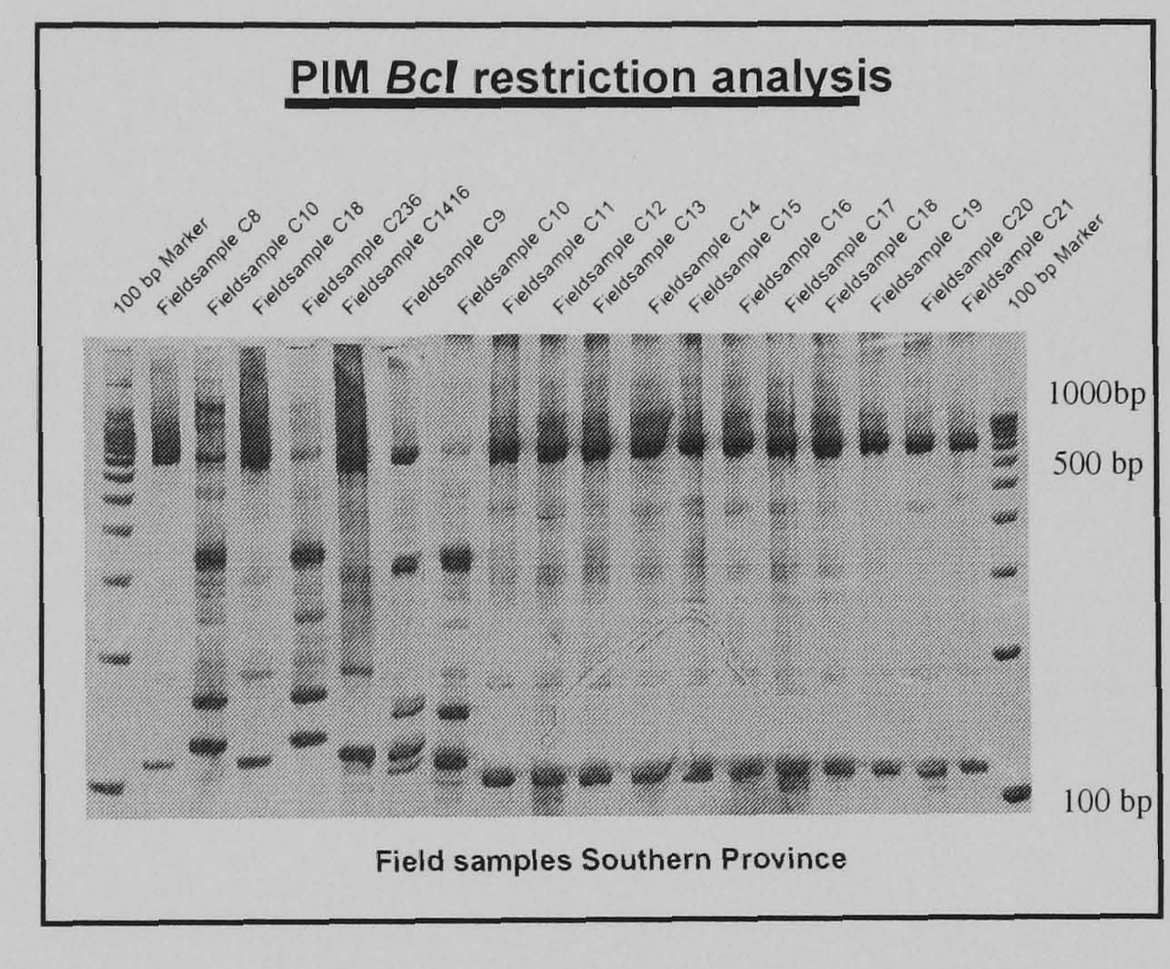

Fig. 44 RFLP-PCR results of fieldsamples from Southern Province using PIM primers. Lane 2 and 6 show mixed PIM genotypes (C10 and 9) and lane 4 a fieldsample (C236) showing a single Kiambu genotype

These results can be split according to the category (clinical status) of the animals sampled. When clinical cases of theileriosis were sampled in the Southern Province, 78% of the samples (n=146) were found to be positive on PCR amplification (Table 15). When sampling of non-clinical cases was done in areas where ECF outbreaks have been previously reported, the percentage of positives was 12% (n=165) (Table 16). The latter samples came from herds located outside the normal ECF infected areas but where veterinary officials suspected that the

reported mortality was caused by ECF epidemics. Subsequent samples from these areas showed an equally low ECF prevalence.

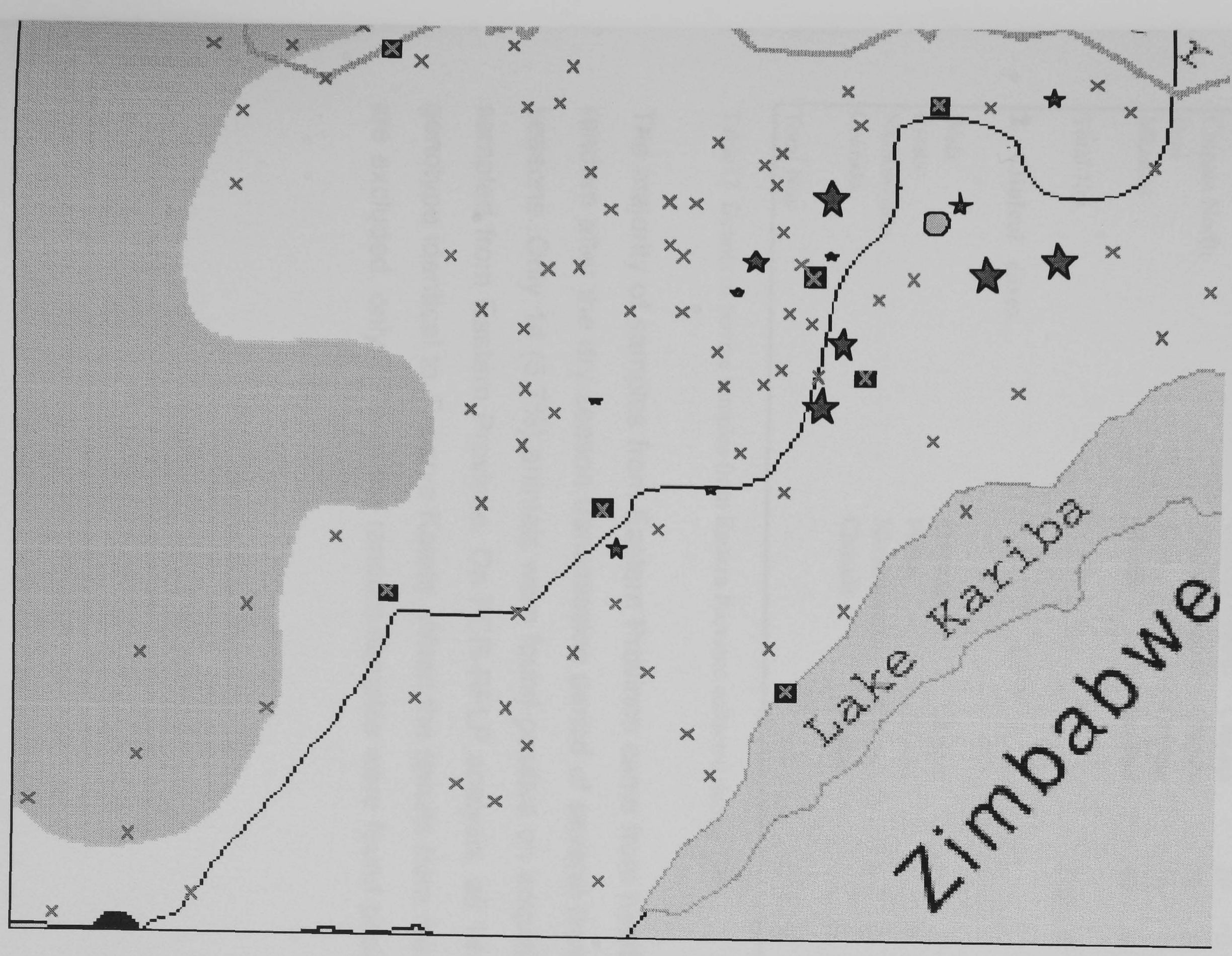

Fig.35. Distribution of T.parva isolates detected in filter paper blood samples from Southern Province of Zambia.
■ District Headquarters; x Crushpens; ★ Mugugatype; ● Southern type

C. Eastern Province random samples

Village/settlement	Crushpen	District	Nos	+ve
1. Random samples				
Wafa	Chiparamba	Chipata	20	0
Quarantine	Quarantine	Chipata	20	0
Nkolowondo	Kamlaza	Chadiza	20	0
Mtenguleni	Mtenguleni	Chipata	20	1
Lufu	Chikando	Chipata	20	0
Mangwe	Mangwe	Chadiza	20	1
Kumadzi	Kumadzi	Chadiza	20	0
Genda	Chipata Central	Chipata	20	0
Chipata North	Chipata Central	Chipata	20	0
Feni	Feni	Chipata	20	7
Mkanile	Mwase	Lundazi	40	0
Total Nrs			240	9
2. Clinical cases				
Wafa	Chiparamba	Chipata	1	1
Pwata	Pwata	Chipata	2	2
Nkolowondo	Nkolowondo	Chipata	1	1
Chanida	Chanida	Chadiza	1	1
Total Nos			5	5

Table17. Details of bovine samples from Eastern Province collected during 1997

The majority of samples from Eastern Province came from healthy animals taken at random after the dry season transmission period of several lower ECF incidence seasons. Only 14 (5.7%) animals were found positive on amplification from the 245 sampled from Eastern Province. On PCR-RFLP analysis, all 14 samples showed a genotype identical to *T. parva* Katete. When the results from the clinical samples are excluded, only 3.7% of the random samples were found positive.

Table 18 gives an overview of the results by origin and category of the samples. Clinical samples originate from sick animals showing symptoms suggestive of a *T. parva* infection (swollen parotid or prescapular lymph nodes). Outbreak samples originate from non-clinical cases in herds were ECF outbreaks have been reported previously. Random samples originate from animals in an endemically unstable ECF area experiencing a low ECF incidence through former large scale immunisations and a suboptimal rainy season.

Province	Samples	Nos	% +ve
South	clinical	146	78
South	outbreaks	165	12
East	clinical	5	100
East	random	240	3.7

Table 18. Overview of general survey results by Province, sample category, number of samples examined and percentage of samples found positive for *T. parva* on PCR amplification.

3.4. Population genetics

The population structure of *T. parva* in the field is derived from the allelic frequencies of the genotypes and the change in their dynamics. The results of the survey in southern Zambia indicate a predominant genotype throughout the area. Because of the presence of one genotype in 96% of the samples, population genetic analysis based statistics become impossible under such an extreme situation (Tibayrenc, 1998).

3.5. Sequencing data on PIM

Four full PIM gene length sequences (*T. parva* Muguga and Marikebuni, *T. lawrencei* 7014 and *T. parva* Boleni) are compared. The PIM PCR assay amplifies between bp 137 and 1032 on the Muguga sequence (total length 1122bp). There are only five point mutations present outside the amplified sequence (Table 19).

	0-132		**PimF**				**PimnF**	**PimR**	**To end**		
Bovine	**A**	**G**	AA	TG	C	G	AC	TC	**A**	**G**	**C**
Buffalo	**G**	**A**	GG		T	C	GA	CG	**C**	**A**	
Boleni				CA	T		AG	CG	**C**	**A**	**G**

Table 19. Point mutations in 5' and 3' primer region of PIM sequence. Nucleotides in bold are outside amplified region (0-132 and from PimR primer sequence to the end). Rest are point mutations within primer sequences.

Analysis of the structure of the PIM gene is performed by comparing sequences of PIM products of various stocks obtained in the nested PIM PCR assay. The cloned products span almost the entire PIM gene. The ORF of the PIM gene in *T. parva* Muguga has been reported to be 1118 bp long (Toye *et al.*, 1991). The products amplified using the nested PIM primers span a region between bp 159 and 1098 of the *T. parva* Muguga sequence, including the first intron. The sequences obtained from the Zam22 and Zam23 stocks were difficult to read and align due to their termination within the repeated sequence. The conserved regions at both ends of the gene and the high proline (P) or glutamine (Q) content of the sequence described by Baylis *et al.* (1993) and Toye *et al.* (1995) were characteristic for all the sequences analysed in this study. The sequences have a GC content of 45%. The amino acid content was more proline (range 14-24%) or glutamine (range 18-25%) rich depending on the isolate.

The PIM sequences have been divided into 17 blocks to facilitate their description (Fig. 46 and for more details, see Fig. 47 and 48). Block Q and part of O are extremely conserved whereas block A contains a few point mutations and one deletion in the buffalo-related sequences. Block B is specific for *T. lawrencei* and buffalo-derived *T. parva* isolates whereas all the other blocks show a large

polymorphism between stocks, although blocks C and F are well conserved among all the bovine isolates. Block H contains tandemly repeated sequences present in the majority of stocks but absent in the short Katete4 sequence or only two repeats long (one cattle and one bovine type) in the Boleni isolates (Table 20).

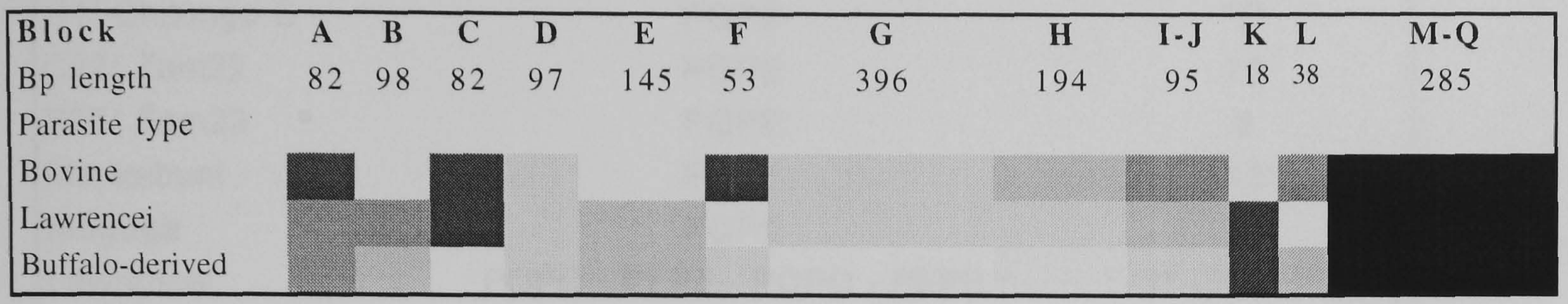

Fig.35. Comparison of polymorphism in blocks of *T. parva*, *T. lawrencei* and buffalo-derived PIM sequences. Darker regions indicate regions of higher homology among the sequences of the different isolates.

An area of PQ-rich, tandemly repeated sequences is present in most stocks towards the 3' end. The repeats consist of the tetrapeptide $(PQPE)_x$ in bovine stocks and $(PEPE)_x$ or $(PQPQ)_x$ in buffalo stocks, and a mixture in buffalo-derived stocks (Table 20). The number of tetrapeptide repeats varies between two and thirteen but there is no correlation between copy number and length of the sequence. The 695bp PIM sequence found in *T. parva* Marikebuni has the highest number of repeats whereas the number of tetrapeptide repeats of the 857 bp long sequence of *T. parva* Katete stock and the short 566 bp of Boleni is very low. Specific buffalo-related regions could be determined which are absent from the cattle sequences. These regions show size and sequence polymorphism among the buffalo-related isolates.

STOCKS	Amino repeat	Length
B2/ Katete	PQPE	2 // 2*
A15/ Katete4 S	PQPE	2
B30/ Chitongo L	PQPE	*7*
C1/ Chitongo S	PQPE	*7*
C22/ Zam22	PQPE	7?
B26/ Zam23	PQPE	8
Marikebuni	PQPE	13
Muguga	PQPE	5*
Lawrencei	PQPQ - PEPE - PQPQ - PEPE	1* - 2 - 1 - 2*
A10/ Zam5	PQPE // PEPE + PQPQ	3* // 4* + 2*
Boleni	PQPE - PEPE	1 + 1

Table 20. Main amino acid repeats and their length in PIM sequences from different *T. parva* stocks. S = short sequence; L = long sequence * 1 amino difference; // not adjoined, ? predicted from poor sequence.

The size of the amplified product can vary from 558 to 954 bp. Short and long sequences can coexist within the Chitongo and Katete parent stocks but in all instances their presence was only revealed after cloning. The relationship between large and short sized sequences has been determined by analysis of the sequence data. They were found to be identical, apart from the deletion of a large segment. The short sizes of the Marikebuni and Boleni PIM sequences are also due to a deletion of a large region. The exact locations where these deletions occur differ but they affect the same zone in the central part of the gene, outside the repeated sequences. Other motifs are repeated one to five times throughout the PIM gene of all stocks (S((X)/G)QQ(G/P)) or a limited number of stocks (VDHQQ(P/Q)). When comparing the buffalo related sequences, an insert (QDDSSGQQQGQQP) was found in block B of the Zam5 sequence partly repeated in block G with no direct relation to other regions (Table 21).

Boleni (block E)	CAGCTAAGGAC	GATCCAAC	CGGACAACAGCAACAACCTCAA	CCTGAACCTGAACCT
Buffalo (block E)	CAGCTAAGGAT	GATCCAAC	CGGACAACAGCCACCACCTCAA	CCTGAACCTGAACCT
Zam5 (block G)	CAACTCAGGGT	GATTCAAG	TGGACAACTAGGACAACAACCA	CCAGTTCAN
Zam5 insert (B)	CAGGAT	GATTCAAG	CGGACAACAAGGACAACAACCA	
Zam5 (block A)			TAGACAACAAGCACAACAACCA	GA
Boleni (block E)	KDDPTGQQQQPQPEPEP			
Buffalo (block E)	KDDPTGQQPPPQPEPEP			
Zam5 (block G)	__DSSGQLGQQPQDQ			
Zam5 insert (B)	QDDSSGQQGQQP			

Table 21. Comparison of nucleotide and AA sequences of the unique partly repeated Zam5 insert and the shared buffalo-related sequences of the PIM region

A large region of sequence homology (GP(V/L)EPVDQ) was shared between Muguga and the buffalo-related sequences in block D. This was followed by similarity in sequences among the Lawrencei and Boleni isolate in block E. Homology among the Zambian isolates and among the buffalo-derived sequences was found in block G. Zambian isolates shared sequences with Marikebuni in the I-J block and the Katete isolates shared a large sequence (PET(T/P)A(P/Q)QEQPQ TPDDQT) with Marikebuni in block L.

Similar relationships could be identified for most blocks of the different PIM sequences to an extent that nearly all blocks could be made up by sequences found in the PIM products analysed.

Sequences were also obtained from recent Zambian isolates, two from Southern (Mazabuka1 and 24) and one from Central Province (Kabwe26). Sequence analysis showed that all these sequences were very similar. The Mazabuka1 sequence was identical to the Muguga sequence apart from probably two point mutations. The Maz24 was identical apart from a three bp insert and two point mutations at different locations. The Kabwe26 partial sequence differed with Muguga by three point mutations.

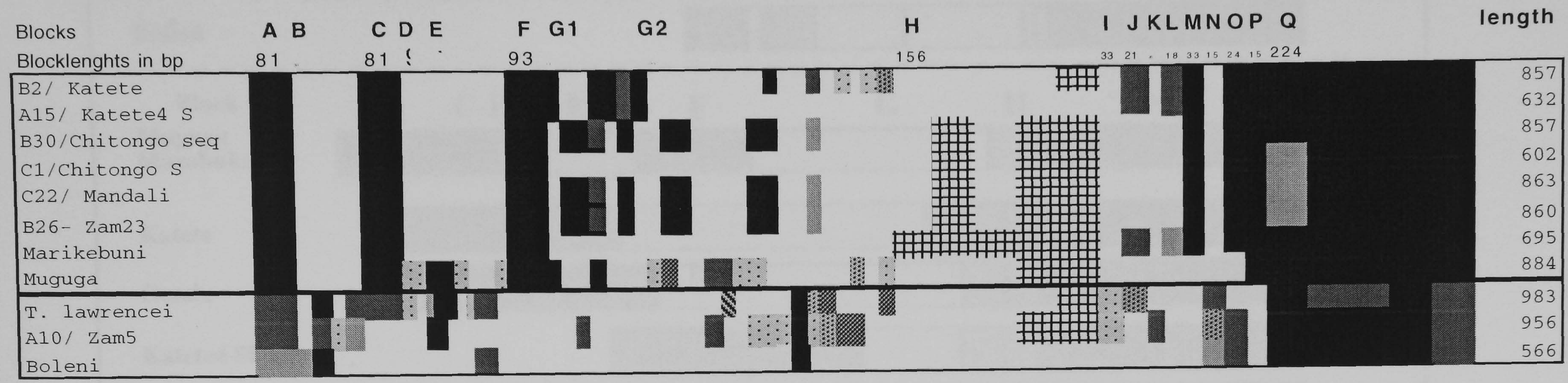

Fig. 47. Sequence comparison of PIM alleles divided in blocks. Dark patterns indicate high % of homology; squares tandemly repeated sequence area.

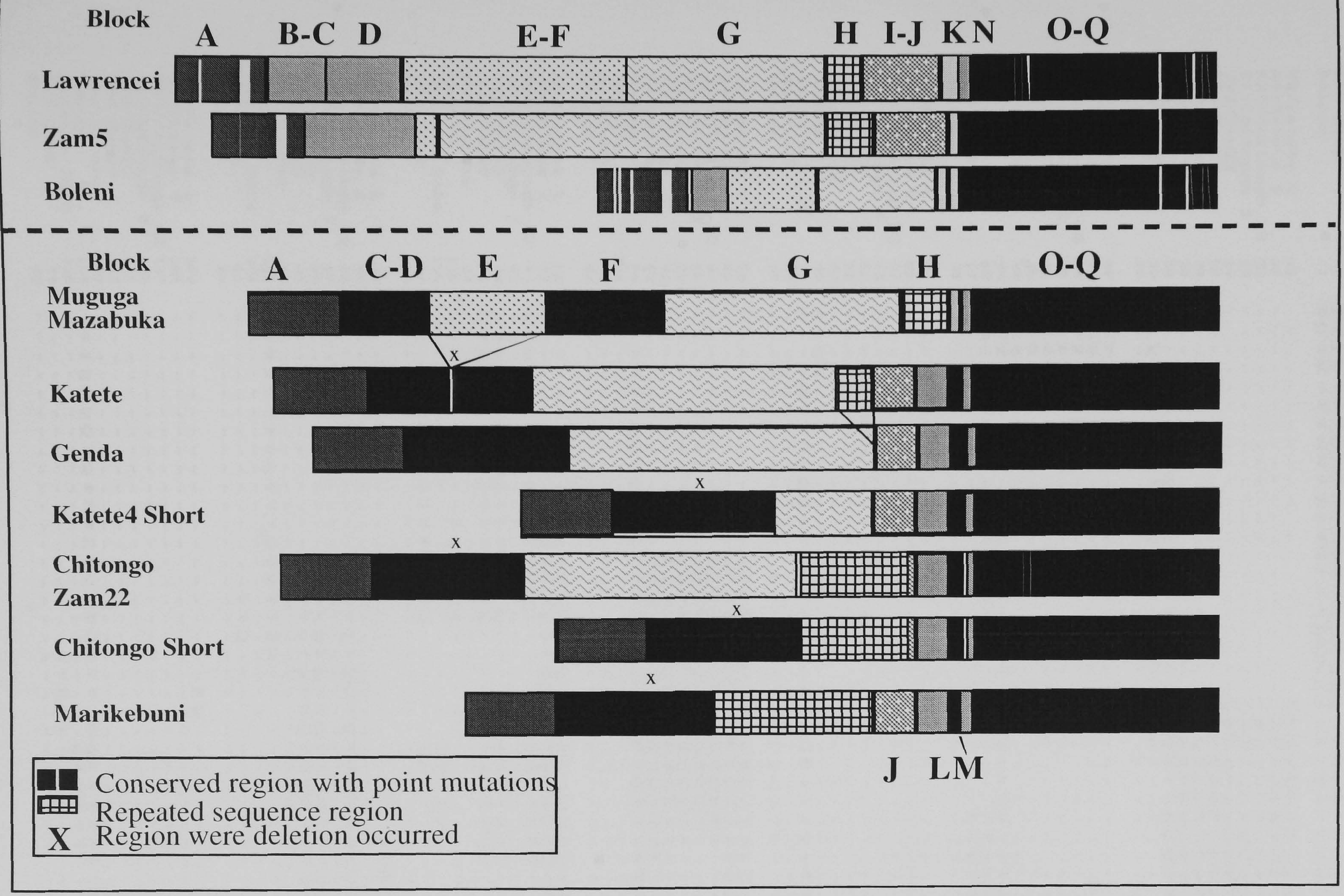

Fig.48. Sequence comparison of PIM alleles divided in blocks. Dark patterns indicate high % of homology; squares indicate tandemly repeated sequence area. Small white lines in conserved regions indicate the occurrence of non-synonymous mutations. The three top sequences are buffalo-derived while the rest are cattle derived sequences. X indicate the place where a large deletion occurred.

Fig. 49. Amino-acid alignment maximilising similarity of PIM sequences from various Zambian and exotic isolates. Shaded areas indicate homology among the majority of sequences, dashes indicate missing amino acids. X is an undefined amino acid, due to a sequencing error.

```
B2/Katete L          1  TQVDTESNDTSSSSETSQQGKPQPDQP----------------  27
A4/Katete S          1  TQVDTESNDTSSSSETSQQGKPQPDQP----------------  27
B30/Chitongo 12      1  TQXDTESNDTSSSSETSQQGKPQPDQP----------------  27
C1/Chitongo S        1  TQVDTESNDTSSSSETSQQGKPQPDQP----------------  27
C22/Zam22            1  TQVDTESNDTSSSSETSQQGKPQPDQP----------------  27
B26/Zam23            1  TQVDTESNDTSSXSETSQQGKPQPDQP----------------  27
Marikebuni           1  TQVDTESNDTSSSSETSQQGKPQPDQP----------------  27
Muguga               1  TQVDTESNDTSSSSETSQQGKPQPDQP----------------  27
Buffalo 7014         1  TQVDTESNDDSSSSETSQQPQQPPDQPVDQQQQPV--------  34
A10/Zam5             1  TQVDTESNDDSSSSETRQQAQQ-PDQPVDHQQQPVQDDS----  37
Boleni               1  TQADSESNDSSSSSETSQQGQQPPIQPVDQQQQPV--------  34

B2/Katete L         28  ----------------------------QDQPDQHQQPTQXDTSG  44
A4/Katete S         28  ----------------------------QDQPDQHQQPTQGDTSG  44
B30/Chitongo 12     28  ----------------------------QDQPDQHQQPTQGDTSG  44
C1/Chitongo S       28  ----------------------------QDQPDQHQQPTQGDTSG  44
C22/Zam22           28  ----------------------------QDQPDQHQQPTQGDTSG  44
B26/Zam23           28  ----------------------------QDQPDQHQQPTQGDTSG  44
Marikebuni          28  ----------------------------QDQPDQHQQPTQGDTSG  44
Muguga              28  ----------------------------QDQPDQHQQPTQGDTSG  44
Buffalo 7014        35  --------QQPSQDQPSGQDSPDQPVDHQQPTQPDQPE  64
A10/Zam5            38  SGQQGQQPETPSQDQPSGQ-----------------------  56
Boleni              35  ----------------------------------------  34

B2/Katete L         45  QQGQQPQ-----------------------------------  51
A4/Katete S         45  QQGQQPQ-----------------------------------  51
B30/Chitongo 12     45  QQGXQPQ-----------------------------------  51
C1/Chitongo S       45  QQGQQPQ-----------------------------------  51
C22/Zam22           45  QQGQQPQ-----------------------------------  51
B26/Zam23           45  QQGQQPQ-----------------------------------  51
Marikebuni          45  QQGQQPQ-----------------------------------  51
Muguga              45  QQGPDTP---QPIQEPSGPVQPDQTGQGPVEPVDQQQQ  79
Buffalo 7014        65  DQQRQQQQPLDQPTGQP---------GPLEPVDQQQQ  92
A10/Zam5            57  ----------------------------GPVEPVDQ---  64
Boleni              35  ----------------------------------------  34

B2/Katete L         52  ----------------------------------------  51
A4/Katete S         52  ----------------------------------------  51
B30/Chitongo 12     52  ----------------------------------------  51
C1/Chitongo S       52  ----------------------------------------  51
C22/Zam22           52  ----------------------------------------  51
B26/Zam23           52  ----------------------------------------  51
Marikebuni          52  ----------------------------------------  51
Muguga              80  PTQG------------------------------------  83
Buffalo 7014        93  P---QPVDQQQPVQEPAKDDPTGQQPPPQPEPEPEPEPEQ  127
A10/Zam5            65  ----------------------------------------  64
Boleni              35  ----------QEPAKDDPTGQQQQPQ--PEPEPEPEQ  57

B2/Katete L         52  ----------DQPVQEQDGQDSQGTPEQTP-----  71
A4/Katete S         52  ----------DQPVQEQDGQDSQGTPEQTP-----  71
B30/Chitongo 12     52  ----------DQPVQEQDGQDSQGTP----DQPQD  72
C1/Chitongo S       52  ----------DQPVQEQDGQDSQGT----------  66
C22/Zam22           52  ----------DQPVQEQDGQDSQGTP----DQPQD  72
B26/Zam23           52  ----------DQPVQEQDGQDSQGTP----DQPQD  72
Marikebuni          52  ----------DQPVQEQDGQDSQGTPE---------  68
Muguga              84  --DTSGQQGQQPQDQPVQEQDGQDSQGTPEQTP-----  114
Buffalo 7014       128  TP----------------------------------  129
A10/Zam5            65  ----------------------------------  64
Boleni              58  TP----------------------------------  59

B2/Katete L         72  ----------DQSGQQPVQQPSGQQQQPQPEPQPEPQ  98
A4/Katete S         72  ----------DQXGQQPVQQPSGQQQQPQPEPQPEPQ  98
B30/Chitongo 12     73  QPDQHXQPTQGDTXGQQ------GQQPQDQ---------  96
C1/Chitongo S       67  ----------------------------------  66
C22/Zam22           73  QPDQHQQPTQGDTSGQQ------GQQPQDQ---------  96
B26/Zam23           73  QPDQHQQPTQGDTSGQQ------GQQPQDQ---------  96
Marikebuni          69  ----------------------------------  68
Muguga             115  ----------DQSGQQ----------------------  120
Buffalo 7014       130  ----------------------ETPPQ----------  134
A10/Zam5            65  -----QQPTQGDSSGQL------GQQP----------  80
Boleni              60  ----------------------------------  59

B2/Katete L         99  P-------------------------------DQPQDQPDQ  108
A4/Katete S         99  P-------------------------------------  99
B30/Chitongo 12     97  ----------PVQEQDGQDSQGTPDQPQDQ---  116
C1/Chitongo S       67  ----------------------------------  66
C22/Zam22           97  ----------PVQEQDGQDSQGTPDQPQDQ---  116
B26/Zam23           97  ----------PVQEQDGQDSQGTPDQPQDQ---  116
Marikebuni          69  ----------------------------------  68
Muguga             121  -PGPDTPDQPVYQQQPVQQ----------------  138
Buffalo 7014       135  ----------------------------------  134
A10/Zam5            81  ----------PVQ-------------------  83
Boleni              60  ----------------------------------  59

B2/Katete L        109  ----------HQ----------------------  110
A4/Katete S        100  ----------------------------------  99
B30/Chitongo 12    117  ----------------------------PDQ  119
C1/Chitongo S       67  ----------------------------------  66
C22/Zam22          117  ----------------------------PDQ  119
B26/Zam23          117  ----------------------------PDQ  119
Marikebuni          69  ----------------------------------  68
Muguga             139  PSGQQQQPQPRPQPQPDQPVDQQQEPPTPEDQPSGPDS  176
Buffalo 7014       135  ----------QEQPTP----------  140
A10/Zam5            84  ----------PVDQK----------QPV  91
Boleni              60  ----------------------------------  59
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B2/Katete L          111  --QPTQGDTSGQQG----------------------------------------  122
A4/Katete S          100  ----------------------------------------------------------  99
B30/Chitongo 12      120  HQQPTQGDTSGQQG----------------------------------------  133
C1/Chitongo S         67  ----------------------------------------------------------  66
C22/Zam22            120  HXQPTQGDTSGQQG----------------------------------------  133
B26/Zam23            120  HQQPTQGDTSGQQG----------------------------------------  133
Marikebuni            69  ----------------------------------------------------------  68
Muguga               177  PDQPD-------------------------------------------------  181
Buffalo 7014         141  ----------------------------------------------------------  140
A10/Zam5              92  EEQTQDDTAGQ---PQPEPQPQPQPEQTPRNTTSTGQP  126
Boleni                60  ----------------------------------------ETPPQQQQPT  69

B2/Katete L          123  ---------QQPQDQPXQEQEGQESQGT----------PE  143
A4/Katete S          100  ----------------------------------------------------------  99
B30/Chitongo 12      134  ---------QQPQDQPVQEQDGQDSQGT----------  152
C1/Chitongo S         67  ----------------------------------------------------------  66
C22/Zam22            134  ---------QQPQDQPVQEQDGQDSQGT----------  152
B26/Zam23            134  ---------QQPQDQPVQEQDGQDSQGT----------  152
Marikebuni            69  ----------------------------------------------------------  68
Muguga               182  ----------------------------------------------------------  181
Buffalo 7014         141  --DDHPSGQQPQDEPVQGPGAQDSPTPDDQPVDQQQPV  176
A10/Zam5             127  TPDDHPSGQQPQDEPVQEQRAQDSPTPDDQPVDKQQPV  164
Boleni                70  PDDHPSGQQPQDEPVQQEQGPQDSP----------  94

B2/Katete L          144  QTPDQ---------------RGQQPXQQPXGQQQQ---  163
A4/Katete S          100  ----------------------------------------------------------  99
B30/Chitongo 12      153  ----------------------------------------------------------  152
C1/Chitongo S         67  ----------------------------------------------------------  66
C22/Zam22            153  ----------------------------------------------------------  152
B26/Zam23            153  ----------------------------------------------------------  152
Marikebuni            69  ----------------------------------------PQ  70
Muguga               182  ---------QHHQPT----------PAAQP--  192
Buffalo 7014         177  HEPVQDQ-------------------------TQPQQ--  186
A10/Zam5             165  HEPVQQEQ------------------------------  172
Boleni                95  ----------------------------------------------------------  94

B2/Katete L          164  ----------------------------------------------------------  163
A4/Katete S          100  ----------------------------------------------------------  99
B30/Chitongo 12      153  ---------PEPQPEPQPE----PQPEPQPEPQPE  174
C1/Chitongo S         67  ---------PEPQPEPQPE----PQPEPQPEPQPE  88
C22/Zam22            153  ---------PEPQPEPXPE----PQPEPQPQPQPE  174
B26/Zam23            153  ---------PEPQPEPQPE----PQPEPQPEPQPE  174
Marikebuni            71  PEPQPEPQPEPQPEPQPEPQPEPQPEPQPEPQPEPQPE  108
Muguga               193  ---------QTQ----------PQPEPQPEPQPE  207
Buffalo 7014         189  ----------------------PQPQPEPQPEPEPG  202
A10/Zam5             173  ----------------------PQPQPEPEPEPEPE  186
Boleni                95  ----------------------------------------------------------  94

B2/Katete L          164  PQPEPQPEPEP----------EPGQEQT----------  181
A4/Katete S          100  ----------EPVQEQT----------  106
B30/Chitongo 12      175  PQPEPQPEPQP----------EPVQEQT----------  192
C1/Chitongo S         89  PQPEPQPEPQP----------EPVQEQT----------  106
C22/Zam22            175  PQXXPQXEPXX----------XPVQXQT----------  192
B26/Zam23            175  PQPEPQPEPQP----------EPVQEQT----------  192
Marikebuni           109  PQPEPQPEPQP----------EPVQEQT----------  126
Muguga               208  PQPEPQ--------------------------------  213
Buffalo 7014         203  Q------QPPVQPVDQQQPVQD----------QPSGKETPQ  227
A10/Zam5             187  PEPQPDQPQDXPVGHQQPTQX----------QPSGQENTQ  216
Boleni                95  ----------------------------------------------------------  94

B2/Katete L          182  ------PETTAQQEQPQTPDDQT----------PEQKPDQPVQ  208
A4/Katete S          107  ------PETTPAQQEQPQTPDDQT----------PEQKPDQPVQ  133
B30/Chitongo 12      193  ----------------------QTPDDQTPEQKPDQPVQ  209
C1/Chitongo S        107  ----------------------QTPDDQTPEQKPDQPVQ  123
C22/Zam22            193  ----------------------QTPDDQTPEXIPXXPVQ  209
B26/Zam23            193  ----------------------QTPDDXXPEXDPDQPVX  209
Marikebuni           127  ------PETTPAPQEQPQTPDDQT----------PEQKPDQPVQ  153
Muguga               214  ----------PE----------PVQ  218
Buffalo 7014         228  PT----------QDDQ----------QVQEPTEQKPE----  244
A10/Zam5             217  PIPDGQ----------PVKEPPEQKPEPE--  235
Boleni                95  ----------IPDDQ----------PVQEPPEQKPEPE--  112

B2/Katete L          209  EPPEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGSG  246
A4/Katete S          134  EPPEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGSG  171
B30/Chitongo 12      210  EPPEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGSG  247
C1/Chitongo S        124  EPPEQAPEHTPSKDDASGEVPVKPSEGHMTGAAADGSG  161
C22/Zam22            210  XPPEXTPXHXPSKDDASGEVPVKPSXGHMTGAAADGXG  247
B26/Zam23            210  VPPDXTPXHTPXKDDASGEVPVKPSEGHMTGAAADGSG  247
Marikebuni           154  EPPEQTPEHTPSPDDASGKVPVKPSEGHMTGAAADGSG  191
Muguga               219  EPPEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGSG  256
Buffalo 7014         245  --PEQTPEHSPSKDDLSGEELVKPSEGHMTGAAADGSG  280
A10/Zam5             236  --PEQTPEHTPSKDDASGEVPVKPSEGHMTGARADGSG  271
Boleni               113  --PEQTPEHTPSKDDASGEVPVKPSEGHMTGAAADGSG  148

B2/Katete L          247  QPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKXTPFHG  284
A4/Katete S          172  QPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHG  209
B30/Chitongo 12      248  QPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHX  285
C1/Chitongo S        162  QPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHG  199
C22/Zam22            248  QPPDKKTDDDSKGKDGSKSGSGTPSKDXKDSKHTPFHX  285
B26/Zam23            248  XPPDKXXDDDSKXKDGSXSGSGTPSKDKKDSKHTPFHG  285
Marikebuni           192  QPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHG  229
Muguga               257  QPPDKKTDDDSKGKDGSKSGSGTPSKDKKDSKHTPFHG  294
Buffalo 7014         281  QPPDKKPDDDSKGKDGSKSDSGTPIKDKKHSKHTPSHG  318
A10/Zam5             272  QPPDKKPDDDXKGKDGSKSGXGTPXKDKKDSKHTPFHG  309
Boleni               149  QPPDKKPDDDSKGKDGSKSDSGSPIKGKKHSKHTPSHG  186
```

4. Discussion

4.1. Protocols and methods of analysis

4.1.1. DNA extraction protocol

The method described by de Almeida *et al.* (1997) was found suitable to amplify *T. parva* DNA from field samples as this study showed that the extraction yielded a positive amplification product from carrier animals in more than 50% of the time. Moreover more than 99% of the field samples yielded a product of the correct size after amplification using the 18S assay. This is proof that the extraction method removes all inhibition present in blood samples (mainly haemoglobin or the anti-coagulant EDTA). It was found that the method is suitable to be applied as a routine extraction due to its ease.

4.1.2. Sensitivity and specificity of the PCR test

The sensitivity and specificity of a PCR reaction is determined by the target sequence and the primer sequences. All primer pairs used produced single bands of predicted sizes from *T. parva* DNA. Detectable size variation was observed with PIM and p67 markers. Specificity was evaluated using DNA samples from the closely related *T. taurotragi*, *T. buffeli* and *T. mutans* species. These samples were always negative after PCR amplification with the different *T. parva* specific primers.

Positive samples are thought to be correlated with the presence of live parasites as it has been shown in rodent malaria infections that only viable parasites are detected by PCR. DNA of injected, killed parasites could not be detected after 48 hrs (Jarra and Snousnou, 1998).

The three assays based on PIM, p104, 18S and p150 antigen genes developed for *T. parva* characterisation produced detectable fragments when between 10 and 30 parasites were present in 5 µl extract used as template in a total volume of 25 µl of PCR mix. This is equivalent to a parasitaemia of 0.001% (1 parasite every 300 microscopic fields) and comparable to the detection of a parasitaemia of 0.00038% in a 100 µl PCR reaction reported for single copy gene PCR in *T.*

annulata (d'Oliveira, 1995) or for a multicopy gene (Tpr1) in *T. parva* (Bishop *et al.*, 1992). The microscopic detection level for Theileria piroplasms can be estimated at 0,003% parasitaemia (1 parasite every 100 microscopic fields). A ten-fold difference in threshold detection among the different loci was found and the highest sensitivity was obtained with the p150 assay. Differences in sensitivity among loci was reported also by Viriyakosol *et al.* (1995) who compared PCR assays of different loci in *P. falciparum*. The amplification results from laboratory-prepared, mixed DNA samples demonstrate the influence of competition between primer molecules for annealing to different targets present in the same sample. It can be concluded that competition between different alleles might interfere with their amplification.

This has been reported before by Contamin *et al.* (1995) for *P. falciparum* infections in which a single or few new variant genotypes, causing a clinical episode, could obscure the detection of polyclonal carrier parasite populations (causing asymptomatic infections). They found that allele ratios below 1/10-1/100 were less likely to be detected. This could explain the failure to detect multiple genotypes in complex parasite stocks, such as in *T. parva* Marikebuni, and the disclosure of new genotypes after cloning of these stocks.

The preliminary results from chronic *T. parva* infections suggest large differences in the frequency of detectable carrier states depending on host parasite interactions and which did not appear to be correlated with time. The parasite densities seem to fluctuate and periodically fall below the level of detection by PCR. Differences between parasite stocks or hosts will influence this pattern being the result of a dynamic equilibrium between the host defences and the evasion mechanisms of the parasite. This equilibrium has been reported to be influenced by differences in physiological state due to nutrition (Chandra and Kumari, 1994) or concurrent infection (Kaufmann *et al.*, 1992).

4.1.3. Methods of sample analysis

Although heteroduplex (HD) analysis is a fast and simple method for comparing two sequences, more quantitative and demanding analyses are required when a range of sequences need to be compared. The HD method generates reproducible results but White *et al.* (1992) reported unacceptable reduction in sensitivity when used on larger (> 400bp) PCR products. SSCP-based analysis has proved very useful in comparing amplification products from different isolates on the same gel but a lack of reproducibility among gels could not be overcome. This is often due to 'smiling' profiles in the gels (Kiyama and Fujita, 1996) interfering especially with characterisation of isolates showing close resemblance in their profiles. Different gels and running conditions were tested to avoid this smiling appearance but none were found to be optimal. Longer sized products, above 400bp, are not considered ideal for SSCP analysis and cutting into smaller fragments by restriction enzymes is normally advised (reviewed by Hayashi, 1991) when looking for point mutations. There was sufficient polymorphism at all three loci used for *T. parva* characterisation so that resolution down to a single base pair mutation was not required when screening for polymorphism among the different amplified products. The SSCP method was still found to be very sensitive and had the advantage of being applicable to fragments lacking sequence data. In all, it was found that the composition of the denaturation mix and running conditions were not the limiting factor in SSCP reproducibility, but it is thought that marked temperature elevation in the gel could interfere with the running conditions. More sequence data of p32 alleles will allow the identification of an appropriate restriction enzyme cutting in the middle region of the p32 sequence. Subsequent digestion of the p32 amplified products before SSCP analysis would resolve the problem of reproducibility.

RFLP-PCR analysis using PAGE gave highly reproducible results and was used as a routine method for characterisation of PCR products of the different loci, provided sufficient sequence information was available to determine relevant restriction enzymes.

4.2. Marker analysis

4.2.1. Characterisation of *Theileria parva* stocks maintained in culture

Southern blot probing results are only available from stocks kept as tissue culture isolates at ITM (all stocks originating from Zambia and the three components of the Muguga cocktail vaccine). This characterisation method requires large amounts of parasite DNA which are only obtainable from *in vitro* cultures. Small amounts of DNA extracts provided by ILRI from exotic stocks (*T. lawrencei*, *T. parva* Boleni, *T. parva* Uganda stocks and *T. parva* Marikebuni stock and clones) are only characterised using PCR-based analysis methods. Southern blot results of exotic stocks, however, are already available from ILRI studies (Conrad *et al.*, 1987 and 1989a; Morzaria *et al.*, 1995; Bishop *et al.*, 1993 and 1994). The Zambian stocks, except the Zam5 stock show very similar profiles with all probes. The Zam5 stock generates profiles which are completely different from all other Zambian stocks. The profiles generated by the exotic stocks, including the Muguga cocktail components, differ markedly from the Zambian stock profiles, except for the Mazabuka stock which is similar to two of the Muguga cocktail components (*T. parva* Muguga and *T. parva* Serengeti-transformed).

The analysis of parasite DNA using different probes and allelic marker assays gave consistent results although marker-based assays gave a higher resolution in differentiating among stocks. Southern blot results using Tpr1 and telomeric probes revealed minor differences between parent and clone-derived profiles. The Tpr1 probe profiles of the Mazabuka clones showed the presence of one band in all clones (Fig.20) that was absent from some temporal isolates of the parent stock. An extra band was found in the Muguga stock after 25 months in culture. The parent and clonal telomeric profiles of Zambian isolates were identical except from the Zam23.H2 clone that had an extra band of 9 Kb. The relevance of these minor differences is unclear. The lack of differences in the profiles generated with the other probes suggest that they are more likely to be the result of a point mutation than of a more important change. The most

remarkable differences were found in the telomeric probe patterns of the *T. parva* Mazabuka isolate and some clones derived from the isolate exhibited both temporal and interclonal variations (Figs.22 and 23). The parent isolate had a fuzzy profile initially which changed back to a Muguga-type profile after 18 months of *in vitro* culture. Its clones had a Muguga RFLP pattern from the beginning of the *in vitro* culture, with no changes occurring over time, apart from some minor size variation in the smaller fragments in the 4 kb range. This is not unusual as telomere regions are known to be prone to expansion and contraction (Pluta and Zakian, 1989; Shampay *et al.,* 1984). The fuzzy Mazabuka parent profile might imply the presence of a mixed genotype but the clones only showed differences in the large 23 kb RFLP fragments. It is unlikely that the superimposition of these clonal profiles would reconstitute the Mazabuka parent profile. It could be that not all components were or could be cloned. If this is the case, the parent profile would be determined by a dominant genotype lacking all smaller RFLP fragments but it is unlikely that this profile would not be cloned. Some physical peculiarities of the telomeric region could interfere with *Eco*RI digestion giving a particular profile, as seen in the case of Zam5. The Zam5 isolate generated the most unique profile using the telomeric probe, showing a condensed one band profile of 23.1 kb resembling the profile generated by the Mazabuka parent stock. The FIGE electrophoresis method was used on several samples, including Zam5, to examine some compacted patterns in more detail. No clear differentiation was possible in the telomeric profiles of Zam5 (Fig.52), although the compacted Tpr1 profiles of the Eastern Province isolates were differentiated into four clear bands. Partial digestion of the DNA with *Eco*RI could be ruled out as identical results were obtained using different extracts and different *Eco*RI enzymes (Bishop, personal communication) and the same Southern blots produced predicted profiles when other probes were applied (Fig. 51). These results may indicate that the eight subtelomeric regions of this isolate are similar in length due to either sequence similarity or to physical peculiarities, concealing distal *Eco*RI restriction sites. The former possibility seems implausible given the marked polymorphic nature of these regions (Morzaria and Young, 1992a).

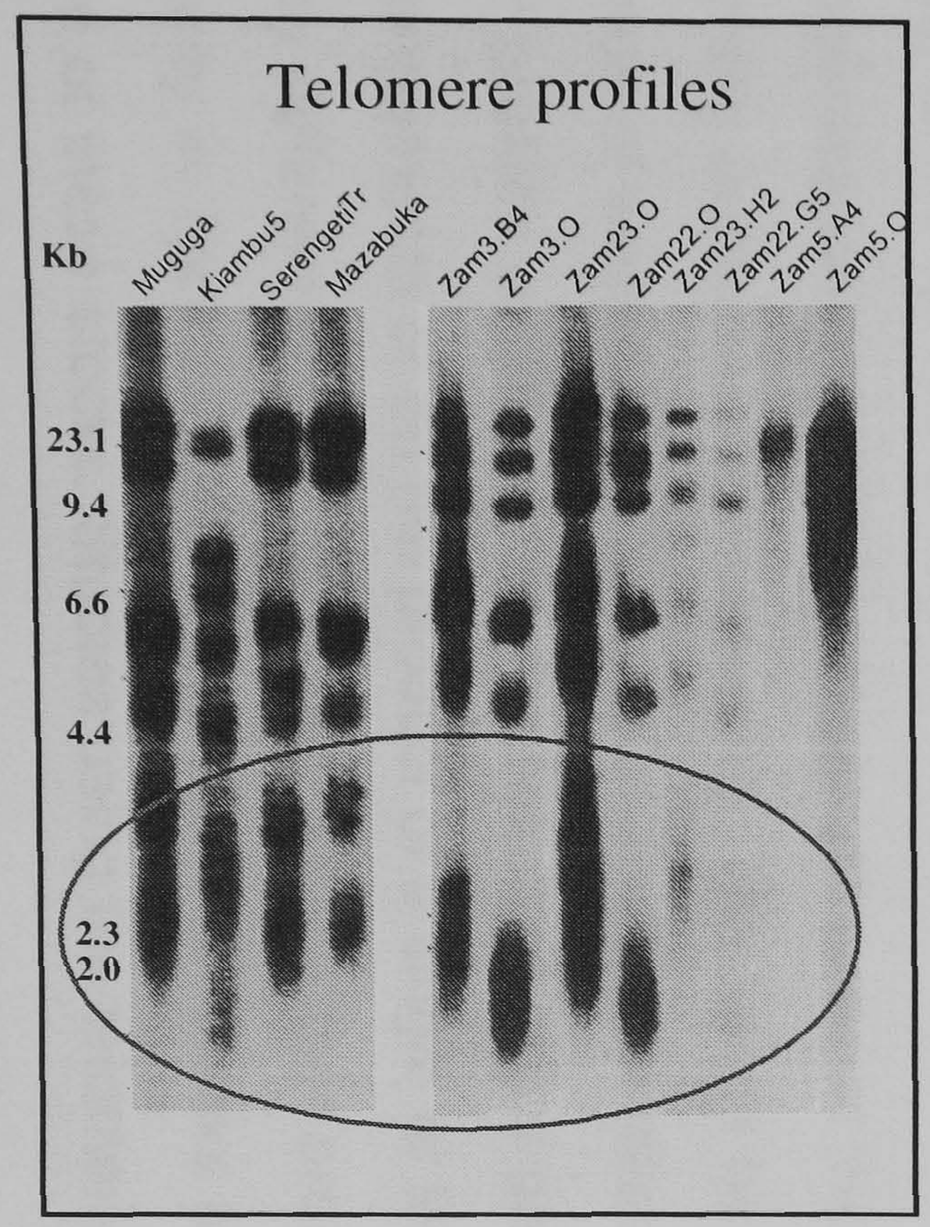

Fig.50. *Eco*RI digested DNA from various stocks probed with the Telomere probe. Highlighted size polymorphism in telomere fragments.

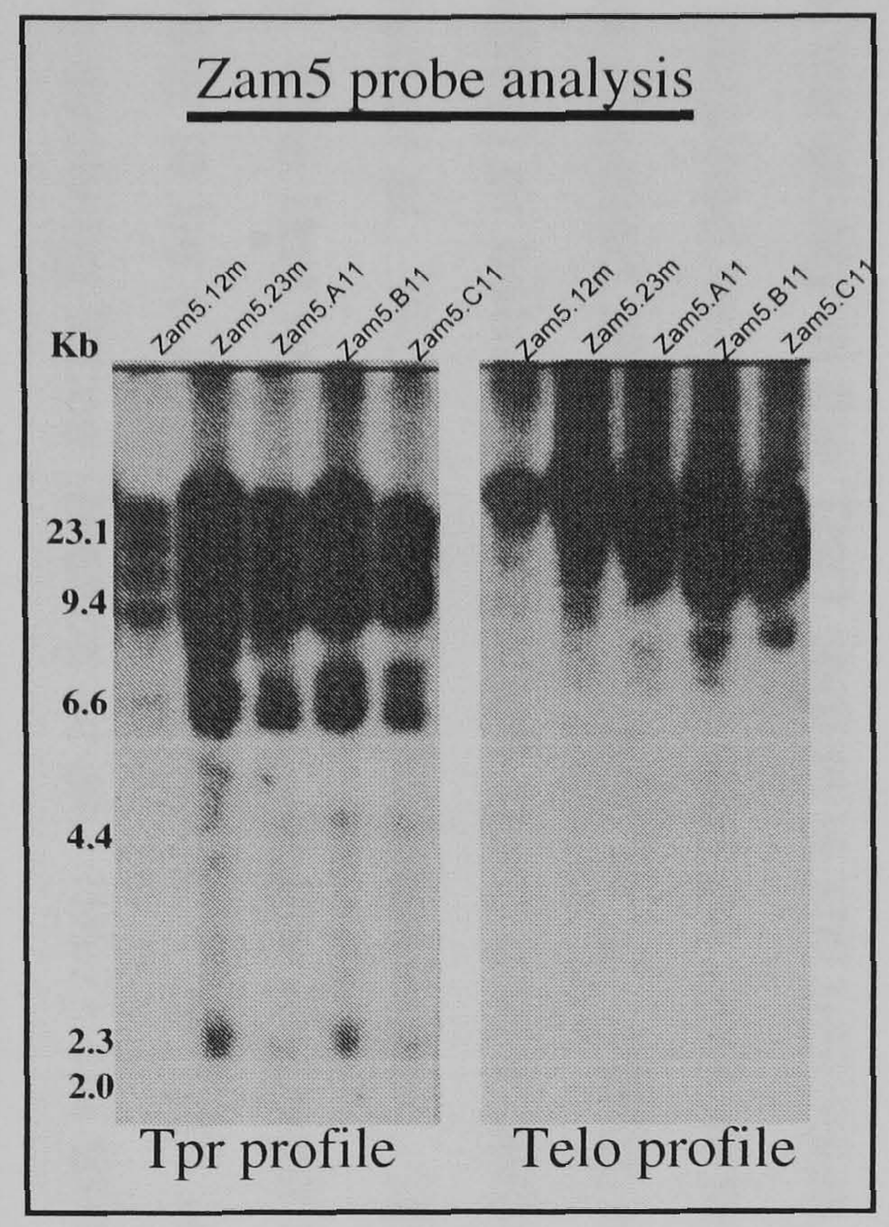

Fig. 51. EcoRI digested DNA from Zam5 probed with TpR1and Telomere probes on the same blot. Comparison between Tpr1 and Telomere profiles of using the same Hybond filter.

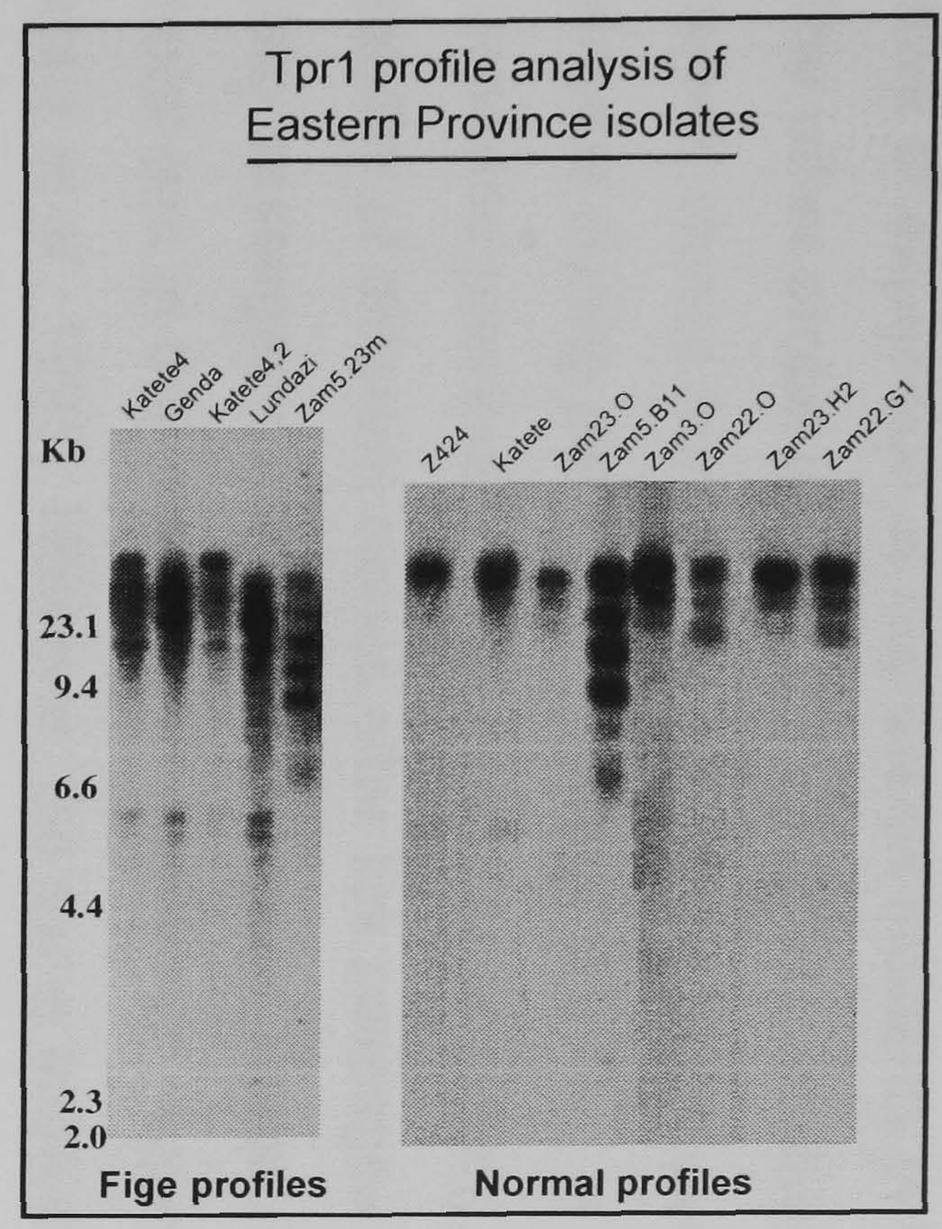

Fig. 52. EcoRi digested DNA from various stocks. probed with TpR1. Comparison between FIGE and normal electrophoresis of Zambian isolates

There was no difference between the parent Zam5 stock and the derived clones, and only minor variation in the thickness of the single band was found over time, indicative of expansion or regression incidents in the telomere sequences (Fig. 50). The other probe profiles did not change between parents and their clones, or between clones from the same parent stock over time, suggesting stability of the genome during passage in culture. Stability in probe profiles of *T. parva* has been reported previously by Morzaria *et al.* (1995). These authors investigated the stability of different clones after tick passages and found identical profiles after two passages using the Tpr1 probe. These data suggested that polymorphism in this region is probably not generated by unequal cross-over (Morzaria *et al.,* 1995). However, the same authors reported on the greater discriminatory power of the Tpr1 repetitive probe in defining different subpopulations in some *T. parva* stocks after cloning. The biological relevance of this variability in the Tpr1 region is still unknown. Slightly different profiles have been obtained using the same probe on DNA extracts from different life-cycle stages (Conrad *et al.,* 1987; Bishop *et al.,* 1993), different passages (Conrad *et al.,* 1989a) or between the parent stock and a derived clone of *T. parva* Muguga (Conrad *et al.,* 1987 and 1989a; Morzaria *et al.*, 1995; Allsopp and Allsopp, 1988). This last report is unusual as the Muguga isolate has been considered generally to be a homogeneous stock. The Tpr1 region is considered as a rapidly evolving locus (Bishop *et al.,* 1997). As this locus consist of repeated sequences and it is thought that mechanisms leading to concerted evolution may be active, it can be expected that unequal crossing over or gene conversion between different genotypes could result in sequence deletion or insertion leading to the spread of slightly different repeated units. On the other hand, homogenisation forces most likely will prevent these newly formed units of becoming numerous subsequently. This might be translated in the presence or absence of weaker bands in Southern blot profiles over time. Differences in RFLP profiles could also be the result of major genomic events such as rearrangements or be the product of a single or few point mutations giving a slightly different profile, especially when other probe profiles are similar. The similarity in Tpr1 profiles of the Zambian stocks highlights the homogeneous nature of these stocks. Studies using the telomeric probe did not reveal

differences between *T. parva* parent stocks and their derived clones, although differences between a *T. lawrencei* parent stock and two derived clones were found (Morzaria *et al.*, 1995). This last result could be explained by the fact that *T. lawrencei* stocks are known to consist of complex parasite populations. Care is needed in interpreting telomeric probe results as these regions have been shown to be prone to expansion or contraction, even during mitosis (Pluta and Zakian, 1989; Shampay *et al.*, 1984) and this has been shown in *Trypanosoma* (Pays *et al.*, 1983, Bernards *et al.*, 1983) and *Plasmodium* species (Dore *et al.*, 1994). These will affect the smaller fragments as in the case of some temporal profile results. Telomere profiles of *T. parva* isolates were very stable over time in this study and could reflect a relative stability of this region as compared to the results obtained with other protozoan parasites. The only exception is the polymorphic profile of the Mazabuka isolate and this supports the suggestion of a different mechanism for this polymorphism.

Southern blot or allelic marker profiles do not always reflect karyotype diversity to the same extent, as has been shown in *Plasmodium* by Druilhe *et al.* (1998) and Daubersies *et al.* (1994). These authors reported on the characterisation of 33 clones derived from two geographically different *P. falciparum* isolates that cause clinical disease. Distinctive karyotypic and phenotypic (drug sensitivity) differences were found within the clones, although the subtelomeric rep20 probe and various allelic markers did not detect differences. Southern blots generate profiles that give an overall view of the genome and are most appropriate in revealing differences due to rearrangements and recombinations, often affecting more than one chromosome. The minor differences found amongst the Muguga, Serengeti-transformed and Mazabuka isolates do not suggest a recombination event but are due to the instability of the loci analysed. The profile results of these three isolates suggest that they are at least closely related genotypes. These results indicate that Southern blot results are relatively stable but that the interpretation of the results should consider the general aspect of the profile due to the less stable nature of the regions used as a marker.

Although karyotype characterisation has not been developed for *T. parva*, the relative genomic similarity needs to be confirmed when additional probes

become available, especially when they relate to the poorly characterised chromosomes one and two.

4.2.2. Allelic marker results

The results obtained by the PCR based characterisation methods on several multi-allelic loci correlated well with the probe results. Other exotic stocks were included in the analysis and results were in line with the known data. The unexpected PIM profile with the buffalo 7014 (Fig. 35) is most likely due to the complex nature of the isolate used. It has been shown repeatedly that buffalo isolates are very heterogeneous (Conrad *et al.*, 1989a; Bishop *et al.*, 1994) The p67 results of the three Boleni DNA extracts (Fig. 42) differed from the results obtained at ILRI. *Theileria lawrencei* and all Boleni stocks gave a 400 bp product indicating the presence of an insert as found in all but one of the *T. lawrencei* stocks (Collins, 1997). The Boleni DNA examined in Antwerp came from an early passage and showed the presence of an insert, whereas the DNA used at ILRI came from a later passage and did not show an insert (V. Nene, personal communication). This result could explain the change in pathogenicity of the Boleni stock during subsequent cattle passages reported by various authors studying different passages (Uilenberg *et al.*,1982; Koch *et al.,* 1988b; Hove *et al.,* 1995) although these reports do not exclude the influence of differences in dose.

The 18S results show clear differentiation between the *Theileria* species and will be of great importance in screening field samples and examining possible interactions. These results also refute a specific species status for *T. lawrencei* and are in line with the sequence results obtained by Collins (1997).

The results of the p32 marker are interesting in that this locus has been studied extensively for most *Theileria* parasites and species specific differences have been detected. These have been used for inter specific differentiation as reported by various authors (Kawazu *et al.,* 1992; Shiels *et al.,* 1995; Knowles *et al.,* 1997). All buffalo-derived isolates showed different SSCP profiles whereas a dimorphism was found in the *T. parva* isolates. The availability of an assay to

differentiate buffalo-derived parasites from cattle *T. parva* would make an important contribution to the study of the epidemiology of *T. parva*.

The Tpr1 amplification gave complex multi-fragment results in Uganda, Marikebuni, Kiambu and Lawrencei isolates contrasting with the single fragment results for most Zambian isolates. The less complex results might be an indication of absence or infrequent recombination rates in these isolates. The PCR results of the Tpr1 primers on the Zam23 stock gave a major fragment of 400bp not present in the other Southern Zambian stocks. Tpr1 amplification of the different clones derived from the Zam23 stock gave a dominant fragment of 1400 bp typical for the Zambian isolates. In some Zam23 clones, the Zam2 parent stock (Southern Province) and the Langa isolate (Eastern Province) a weaker amplification product or products were revealed similar to the 400bp fragments in the Zam23 parent stock. These results are difficult to explain. The smaller fragments obtained after PCR amplification of some Zambian isolates might be the result of priming of a related sequence or the result of an earlier recombination event. The size similarities of these minor fragments suggest a common event. It is unlikely that the polymorphism in the Tpr1 amplification products of some Zambian stocks could be related to major recombinations at this locus as the Southern blot results with the Tpr1 probe refute such hypothesis. Tpr1 sequences are 1.4 Kb long repeated sequences with a high copy number (100 copies per genome) revealing conserved regions in several analysed *T. parva* isolates (Baylis *et al.*, 1991) and Tpr1 priming results in this study have confirmed this observation. One of the explanations for the Zam23 results could be the presence among Zambian isolates of different gradations in homogenisation of a common ancestral rearrangement event. Due to the Zambian epidemiological situation resulting in low recombination rates it might take a long time before homogenisation of all repeated units is completed. This would be unlikely as under this hypothesis it would mean that the Zam23 parent stock contains a major population undergoing a different homogenisation process compared to the other Zambian stocks as hardly any fragment was amplified. An alternative hypothesis could be the presence of a related sequence to one of the Tpr1 primers in the isolate specific region of Tpr1 giving a large and a smaller fragment. The results of the Zam23 stock should than be

interpreted as atypical and the result of preferential priming of the related sequences of a major population not adapted to the *in vitro* culture system as reflected by the absence of this genotype in its derived clones. This related sequence could be present in low numbers in other Zambian stocks as indicated by the Tpr1 priming results of the Zam23 clones, some Zam2 stocks and the Langa stock. Sequence data of Zam23 cloned PCR products could clarify the latter hypothesis. It should be noted that the latter stocks could not be differentiated from the other Zambian isolates using the other allelic markers or probes. The Tpr1 analysis of both Southern blots and PCR amplification indicate that the complexity of this locus prevents an easy interpretation and caution for the use of this region in characterisation of isolates. More research is needed for better interpretation of the informative power of this region.

It can be concluded that the Zambian isolates, although limited in number but isolated from different districts, are very homogeneous and related to each other. Differences on Southern blot profiles were related to the presence or absence of one band. The notable exception was the Zam5 isolate which shows a distinct relationship with *T. lawrencei* parasites on all allelic markers. There was no indication of multiple parasite genotypes within this isolate, although selection during *in vitro* culture could not be ruled out. This has been described for *Plasmodium* (Viriyakosol *et al.,* 1994) and was reported as a possibility in one *T. annulata* study (Sutherland *et al.,* 1996). If the presence of different genotypes was obscured by one dominant type, the cloning of stocks should reveal other genotypes, which was not the case in this study.

This is in marked contrast to the heterogeneity of profiles detected in East African stocks (Conrad *et al.,* 1989a; Bishop *et al.,* 1994). The complexity of the Marikebuni stock (four different genotypes) was confirmed by multi-locus analysis but does not seem to be generated by recombination between the various loci of the different components. In fact, the majority of multi-locus genotypes show a linkage between the p150 and p104 locus, although these are located on different chromosomes. This could be another indication of low recombination rates in *T. parva* parasites as has been reported before by Bishop *et al.,* 1993.

Conrad *et al.* (1987) found evidence for the presence of different parasite genotypes in *T. parva* Marikebuni DNA by Tpr probe studies. This is in sharp contrast with results obtained from *T. parva* Muguga, another Kenyan isolate, showing no Mr variation of the PIM protein (Toye *et al.,* 1991). Cross-immunity results using *T. parva* Marikebuni cloned parasites showed that animals were not always completely protected against the parent strain challenge (Irvin *et al.,* 1983; Morrison *et al.,* 1989). These results provided strong evidence for a multi-component structure of the *T. parva* Marikebuni isolate. Although not cloned, *T. parva* Muguga is considered a homogeneous strain although some heterogeneity at the DNA level was reported by Conrad *et al.* (1987) and Morzaria *et al.* (1995) using Tpr probes. The homogeneity is supported by results from cross-immunity studies with *T. parva* Muguga clones and the parent stock, showing no breakthrough reactions (Goddeeris *et al.,* 1990).

It can be concluded that the results obtained using the allelic marker assays are in close agreement with the Southern blot results. Although the latter display a higher degree of polymorphism, the relevance of this polymorphism does not seem to be correlated with major parasite populations but more with individual variation amongst parasites. In other words, the level of resolution is too high to be used for the characterisation of populations. But it is not certain if these minor differences (often limited to one single band) among similar Southern blot profiles are mirroring phenotypically relevant differences. Multi-locus genotyping using the allelic marker assays, based on genes, has the advantage of ease of interpretation and the information obtained is correlated with changes in relevant sequences. However, the number of allelic markers should be increased to give a more complete picture of changes over the whole genome (preferably including markers on chromosomes one and two).

4.2.3. Field survey results

4.2.3.1. Field survey results using Southern blot characterisation

The probe results of the field isolates from clinical cases obtained from the Eastern and Southern Provinces before immunisation trials were conducted are similar, except for the southern isolate, Zam5. These results suggest the

presence of a limited number of strains and a common origin for the majority of isolates from both Provinces. The better discrimination obtained with the LA6 probe is in agreement with a previous report (Nene *et al.*, 1998) and is of interest as this probe is derived from a multicopy locus containing an ORF in its sequence. The LA6 locus is transcribed in the schizont and piroplasm stages (Bishop et *al.*, 1993a) but neither its full genetic characteristics nor its function are known.

The results of this study resemble Southern blot results of *T. parva* isolates obtained from clinical cases in a study of parasites from Zimbabwe (Bishop *et al.*, 1996). The *T. parva* epidemiology in Zimbabwe has been characterised as an epidemic unstable disease situation (Koch, 1990; Norval *et al.*, 1991). The characterisation of parasites was done on DNA extracts from schizont-infected cell lines isolated from clinical cases in the field. The Southern blot results obtained using three probes revealed a generally homogeneous population structure in cattle-derived *T. parva* parasites from the highveld (Bishop *et al.*, 1996) with identical Tpr1 profiles. This correlates well with the results of cross-immunity trials reported by Koch *et al.* (1990) with isolates from the same region. Koch *et al.* (1990) suggested that there was a limited number of antigenically different parasite types in Zimbabwe. These results are in marked contrast to data obtained from isolates originating from Kenya that show complex *T. parva* populations (Allsopp *et al.*, 1989; Conrad *et al.*, 1989a; Toye *et al.*, 1991; Bishop *et al.*, 1993a and 1993b). The probe profiles resulting from the characterisation of buffalo-derived parasites in the Zimbabwe study showed a marked heterogeneity (Bishop *et al.*, 1996). This is in agreement with the results of studies of *T. lawrencei* isolates from Kenya and Tanzania (Conrad *et al.*, 1987 and 1989b).

A similar complex situation has been reported for *T. annulata* isolates from the field in Tunisia (Ben Miled *et al.*, 1994). Characterisation of tissue culture isolates was attempted using different molecular-based methods. Southern blotting with two probes (TaT17; TaT21), glucose phosphatase isomerase (GPI) patterns and mAb typing (mAb 7E7) revealed extensive genotypic heterogeneity among parasite populations and even within the same isolate. No isolates out of a total of 53 showed the same profile using a combination of markers and up

to four variants were detected within one parasite isolate. This heterogeneity was not correlated with geography as the same diversity was found among samples from the same farm. Despite the complexity at this level, the protection provided by one stock is broad (Pipano, 1981; Brown, 1990). The complexity of parasite populations in *T. annulata* could be explained by biological differences related to sexual reproduction of this parasite. In contrast to *T. parva*, the point at which meiosis takes place has not been determined. The presence of multiploid kinetes in the salivary glands could mean that meiosis occurs after entry of these multiploid kinetes into the acinus of the tick (Gauer *et al.,* 1995). This could create a complex parasite progeny in a single acinus in the case of zygote formation between a mixture of parasites from two strains. This means that the majority of animals infected by ticks showing a single infected acinus could acquire mixed infections.

Other factors related to lifecycle differences, transmission intensity or differences in vectorial capacity of the tick species might also influence this complexity. The fact that different cell populations are invaded by sporozoites of the two parasites could reflect differences in survival possibilities of the two species and could lead to a distinct biological evolution as suggested here. The epidemiology of *T. annulata* in Tunisia has been described by Darghouth *et al.* (1996) and only four farms were categorised as endemically stable according to end of transmission season sero-prevalence. These farms showed high tick infestation rates of over 50 ticks per adult host whereas less than 50 ticks were found feeding on an adult animal in most areas. Sero-prevalence was between 50% and 100% in the endemically stable farms and between 20% and 30% in the endemically unstable farms in all age classes, after the second transmission season. Moreover, low *Theileria* prevalence rates and low infection intensities have been reported in *H. detritum* ticks in this region (Bouattour *et al.,* 1996). This could exclude the influence of transmission intensity or vectorial capacity differences on the complexity of *T. annulata* populations.

4.2.3.2. Field results using allelic markers

The same Zambian isolates were characterised using six allelic markers and the results are in agreement with the results obtained by Southern blot analysis. The Tpr1-based PCR gave a distinct band at 1400 bp for all Zambian isolates, except for Zam5. This assay was efficient in differentiating the Zambian from the non-Zambian isolates. The PIM assay was the most effective in differentiating among the Zambian isolates within one Province. None of the isolates showed a mixture of genotypes in any of the allelic-based assays. These results highlight the value of the PCR assays in characterisation studies and underline the limited diversity, and the particular identity of the majority of *T. parva* stocks in Zambia, before immunisation was initiated. The different percentages of positive samples found in the Southern Province surveys are related to the geographical origin of the samples and the time of collection. The sampling of clinical cases was done in areas where regular ECF outbreaks are experienced and took place during the ECF peak incidence in the dry season whereas the sampling of suspected ECF recovered animals was done outside the regular ECF-infected region and mainly during the lower incidence period in the rainy season.

The use of PCR based assays in characterisation of field parasites in endemic *P. falciparum* regions have been reported by various authors (Scherf *et al.,* 1991; Mercereau-Puijalon *et al.,* 1991; Daubersies *et al.,* 1994; Contamin *et al.,* 1995; Viriyakosol *et al.,* 1995). The results, based on multi-locus characterisation with a maximum of four loci, revealed an enormous diversity in *P. falciparum* populations. The majority of isolates from some regions contained unique parasite genotypes with regard to polymorphic single copy genes (Conway and McBride, 1991; Contamin *et al.,* 1996 and Kyes *et al.,* 1997b). These results highlight the possibilities of PCR-based allelic typing studies in field investigations and this approach is proving very successful in population genetics studies and epidemiological surveys. However, the presence of different genotypes within the same sample usually creates a complex situation, not only in relation to genotype amplification, but also to correct identification (Druilhe *et al.,* 1998; Ferreira *et al.,* 1998). This complexity was also demonstrated when cloning of PCR products of the PIM locus revealed a mixture

of two different alleles in one Eastern (Katete) and one Southern Province isolate (Chitongo). The biological relevance is difficult to determine as the sequences of these allele mixtures were similar apart from a large deletion in the central region of the smaller alleles.

4.2.3.3. p32 results

Two different alleles have been identified in the different *T. parva* isolates analysed. All Zambian isolates, the Muguga and the Serengeti-transformed stocks showed an identical SSCP p32 profile, indicative of a highly conserved gene. On the other hand, the results of SSCP analysis on various buffalo-derived stocks gave different profiles from those found for *T. parva* and the profiles were heterogeneous among the stocks. The preliminary results from studies of the p32 locus in three *T. parva* isolates (Muguga, Katete and Chitongo) using SSCP analysis and sequence data suggest a highly conserved locus. The conservation of the p32 gene in *T. parva* as opposed to the polymorphism found in buffalo-derived isolates highlights again the difference between cattle and buffalo-derived parasites and their role in the ECF complex. The biological relevance of the conservation of p67 and p32 in cattle-derived *T. parva* is not known. Both genes code for surface proteins of invasive stages of the parasite and might reflect important functional restrictions in host cell interaction leading to the absence of polymorphism in these ligands.

In contrast, a study of *T. annulata* diversity using a Tams1-1 based probe of the related p32 locus on various isolates from Tunisia and Turkey and one Indian isolate revealed polymorphism that was not related to geography (Shiels *et al.*, 1995). Sequences from five geographically different isolates showed a close relationship, although significant variability was found within a specific region of the coding area where the majority of N-linked glycosylation sites were located (Shiels *et al.*, 1995). Over 25 alleles have been sequenced and predicted amino acid alignment has revealed three short polymorphic and several conserved regions interspersed with amino acid substitutions throughout the gene (Katzer *et al.*, 1998). There is evidence for intragenic recombination between Tams1 alleles (Katzer *et al.*, 1998). The discovery of a hybrid sequence suggests the

occurrence of genetic recombination via crossing over and could explain sequence relations between different Tams alleles (Shiels *et al.*, 1995). Evidence for random assortment of allelic markers following co-transmission of *T. annulata* clones through the tick vector has been reported (Shiels *et al.*, 1995). The polymorphism found in the p32 homologue in *T. annulata* is intriguing and reflects a fundamental difference in host interaction mechanisms of the two parasites. Schizonts are the pathogenic stages in *T. parva* whereas the piroplasm is the pathogenic stage in *T. annulata*. It could be that host responses to the pathogenic parasite stage will be more critical for parasite survival leading to positive selection in the merozoite protein coding for an erythrocyte ligand.

4.2.3.4. Survey in the Southern Province of Zambia

The results of the survey of the *T. parva* parasites in the Southern Province of Zambia following the application of immunisation clearly demonstrate a dominant genotype in the bovine population that was similar to one of the components of the cocktail vaccine parasites. In fact, all samples positive for this genotype showed identical profiles for the three allelic markers, highlighting the stability of these markers and their suitability for characterising field populations. The low prevalence of mixed genotypes (0.6 %) points to a less complex population structure than usually found in *Plasmodium* or *T. annulata* field surveys. It could be anticipated that cocktail-type vaccines provide an ideal opportunity for mixed genotype propagation. In fact, what was found was a low percentage of mixed genotypes and an absence of recombinant genotypes. Under laboratory conditions, recombination between co-infecting stocks has been demonstrated and 20% of the progeny (S. Morzaria, personal communication) showed extensive recombination between various chromosomes (R. Bishop, personal communication). The low frequency of mixed genotypes in the field in Zambia could be explained by differences among the stocks in multiplication rate, parasitaemia patterns, carrier potential or vector infectivity, or a combination of these. This would be sufficient to allow selection over time of the parasite population best adapted to the prevailing transmission conditions. The absence of recombinants could be related to low fitness of

these new genotypes in comparison to the wild genotypes. On the other hand, simulation models described by Hastings and Wedgwood-Oppenheim (1997) predicted a significant time lapse between introduction and appearance of recombinants. It might be too early to find recombinant types as they might be present in the sample at too low a frequency to be detected. Most of these hypotheses could be tested but the confounding effects of the different factors listed above might be much more difficult to unravel. The Muguga component of the cocktail does not induce a carrier state (Young *et al.,* 1990, Bishop *et al.,* 1992), the parasitaemias induced by each of the three stocks in the cocktail vaccine have been found to be similar in experimental infections, although this may vary if the initial dose varied. These might differ as the cocktail vaccine stabilate doses are generally adjusted to LD_{50} values and not to sporozoite numbers. The parasitaemia of the different parasite components may vary even after delivering equal numbers of sporozoites, as host interactions at various stages might contribute to differential development. This would influence the potential of each of the three stocks to contribute to the piroplasm mixture in the blood meal of the tick.

The fact that a genotype, identical to these found in pre-vaccination era isolates, was only detected in two samples in the 1996 survey suggests that a novel genotype was introduced by vaccination that subsequently became established and caused large epidemics in the province. An alternative hypothesis is that an existing genotype, similar to *T. parva* Muguga was present but had not been isolated and later became the dominant genotype can not be ruled out. There is some indication from the results of this limited survey that PIM genotypes might be geographically correlated and further work is planned to clarify this relationship.

A *P. falciparum* survey of symptomatic patients in French Guyana (Ariey *et al.,* 1997) provided similar results to these found for *T. parva* in southern Zambia. The epidemiology of malaria described in this paper was exceptional with year round transmission but very low inoculation rates (incidence rate of 0.1%). This resembled the southern Zambian situation with low inoculation rates and limited polymorphism. Although mixed *Plasmodium* infections were found in 12% (n = 125) of the cases, this was associated with a very low polymorphism in the field

parasite population (four different alleles for MSP-1, two for MSP-2 and four for the GLURP locus were found) (Ariey *et al.,* 1997). Using a computer simulation model, a correlation was shown between prevalence and stability of polymorphism. A threshold of 25% prevalence was predicted to maintain stable polymorphism. When prevalence dropped below this threshold, genetic drift would result in the loss of certain alleles and the fixation of others, thereby decreasing polymorphism. This appears to correlate well with the PCR results in this study in which prevalence rates were found to range between 3.7 and 12 %.

Clinical and parasitological data accompanying the field samples indicated that the *T. parva* Muguga genotype was associated with cases showing high parasitaemias and mortality. The stabilates of two *T. parva* stocks from Southern Province, isolated before the introduction of the cocktail vaccine produced consistently low parasitaemias in naïve *Bos taurus* experimental animals (Musisi, 1986, Asveza South and own results). The high parasitaemias seen and the predominance of the Muguga genotype in samples of clinical cases strongly suggest that the Muguga genotype is the causative agent of the ECF epidemics in the Southern Province of Zambia. That such a situation could arise can be explained by the epidemic nature of ECF in the Southern Province, as has been reported earlier by Moorhouse *et al.* (1985) and confirmed by Mulumba (1997). The high numbers of animals remaining naive under an epidemic disease situation would facilitate the introduction of vaccine stocks as the immunisation was done on an 'ad hoc' basis and only calves were included in the vaccine programme. This leaves a large proportion of the herd susceptible to infection and creates ample opportunities for expansion of newly introduced isolates, even in the case of cross-immunity between these and the local isolates. It can be concluded from the results that the vaccine stock is a more successful parasite than the wild type under the conditions existing in that environment. In view of the recent history of ECF in Southern Province both hypothesis are likely.

If a *T. parva* (Muguga-type) parasite has become established as the dominant type, how has that happened if one of its characteristics is that it does not

produce a carrier state (Young *et al.*, 1990, Bishop *et al.*, 1992). Three hypothesis can be considered that might explain this situation.

- The *T. parva* Serengeti-transformed parasite component of the cocktail is producing a carrier state. This would be unlikely as the Muguga and Serengeti genotypes are nearly identical, implying a common descent and the likelihood that both stocks lack a carrier state. The Muguga and Serengeti-transformed components differ only in their Tpr1 profiles. This region is considered to be a rapidly evolving locus and has shown slight differences in profiles at different life-cycle stages or passages using the same stock (Conrad *et al.*, 1987 and 1989a). The almost identical Tpr1 profiles (differing in one band) and the identical results of all other probes and markers suggest a very close relationship between these two stocks. Moreover, there is strong evidence from mAb profiles and cross-protection data, of a superinfection with a *T. parva* Muguga type parasite during the transformation experiments leading to the*T. parva* Serengeti-transformed stock (Reviewed in this thesis under 1.1.1.).
- Their has been recombination between *T. parva* Muguga and one of the vaccine stocks or a local stock. No evidence for this was found in the characterisation data using genome-wide probes. Three isolates in total made from clinical cases of ECF in the Southern and Central Provinces showed exactly the same genotype as *T. parva* Muguga or *T. parva* Serengeti-transformed, but no recombinant genotype was found using the three allelic markers.
- A more probable explanation may be found in the ecological and epidemiological differences between the Eastern and Southern Provinces.

Disease prevalence	Rainy season	Dry season	Tick ecology
Southern Province			
1996	5	27	
Eastern Province	65	35	2 generations
1995	44	13	1 generation
1996	8	6	

Table 22: Clinical cases in % during rainy and dry season in both ECFaffected provinces of Zambia.

There is a markedly lower incidence of clinical cases during the rainy season in Southern Province, reflecting a lower infection rate in AA ticks. The climatic conditions differ between the provinces in that the minimum temperature is lower during the cold season in the South and the start of the rains is 2-3 weeks earlier. This results in an earlier start of the tick life cycle with LL appearing around mid February in Southern Province (data from development studies). These LL could become infected on *T. parva* Muguga clinical cases which generally show high parasitaemia. Animals that recover from *T. parva* Muguga infection are probably infective for up to 2.5 months (S.P.Morzaria, personal communication) before the infection is eliminated.

The peak of clinical disease in the Southern Province is in May-June, during the peak of nymphal activity (see Table 22). This has been attributed to an important LL role in picking up infection during the rainy season (Mulumba, 1997). Although experimental data on LL vectorial capacity shows low infection rates in resulting NN, using local LL with local *T. parva* isolates (Ochanda *et al.,* 1996) but when isolates from outside the region are used, the resulting infection rates in NN ticks are much higher. An higher infectivity of *T. parva* Muguga in the Southern Province ticks could explain the epidemiological findings in Southern Province (Ochanda *et al.,* 1998; Tempia, 1997).

The presence of nymphal ticks during the period of high clinical disease incidence in May-June will ensure pick-up from reacting and early recovering animals infected with a non-carrier *T. parva* infection. This infection will be carried over in the tick to the next rainy season.

4.2.3.5. Survey in the Eastern Province of Zambia

Results of the survey of genotypes from the Eastern Province revealed a dominant genotype related to the local *T. parva* Katete stock that has been used as a vaccine since 1986. The low number of positive samples was unexpected as it was anticipated that there would be many carrier animals (Asveza Project and own data) following immunisation which should lead to a year-round presence of detectable parasites in more than 50% of the cattle. The fact that

samples were taken after the transmission period would not explain this discrepancy, although similar findings have been reported in malaria research. Kyes *et al.* (1997) reported a higher incidence in asymptomatic carriers (10%, n=50) at the start of the transmission period than towards the end (0.8%). It has been observed in tick transmission experiments (Young *et al.*, 1986) and using PCR amplification (Bishop *et al.*, 1992; own results) that parasite levels in carrier animals tend to fluctuate. Data from experimental studies following chemotherapy (Dolan, 1986a and 1986b) also indicated an intermittent transmission from recovered animals with predictable patterns for particular parasites. It cannot be ruled out that under natural conditions circulating parasites in carriers might be below the PCR-detection threshold. Moreover, there are no data available on the influence of different cattle breeds on the dynamics of the carrier state and the levels of parasitaemia. The use of immunisation and the number of immune animals in the Province will contribute to a decrease in the occurrence of acute infections with high parasitaemias. And little is known about the influence of rechallenge infections on the carrier state. These findings highlight areas where more research is needed in order to develop accurate transmission models based on well designed field studies and data generated using molecular tools.

4.3. Population Genetics

Allele over-representation could be the result of bias in the analysis due to the small number of markers used and or limited number of samples. The sample size used would not bias the results, as sample sizes over 100 are considered adequate for meaningful analysis (Tibayrenc, 1995, Ferreira *et al.*, 1998, Paul and Day, 1998). There is no doubt that the use of more markers would increase the validity of the results and might add discriminative power. Nonetheless, there is good evidence for the effectiveness of the PIM marker in distinguishing the genotypes of different clones derived from the *T. parva* Marikebuni stock (Fig. 36 and Table 14). This marker would pick up most mixed genotypes present and the combination of the three markers would characterise and differentiate among a fair number of recombinants because of their location on different chromosomes. On the other hand, the amplification of only one genotype from

the *T. parva* Marikebuni parent stock by PCR was unexpected and prompted further research on DNA template mixtures. This showed that competition for primer annealing between different genotypes in the same PCR mix might interfere with optimal amplification of certain genotypes. Differences in genotypes frequencies above 1:10 would probably result in non-amplification of the least frequently present template(s). The Marikebuni clones and clones from two Zambian isolates had genotypes that were not revealed in PCR amplifications from the parent stock. The Marikebuni stock has been shown in various studies to consist of different genotypes (see section 1.2.4). The results obtained with the PIM assay suggest that one genotype is predominant in *T. parva* Marikebuni. Cross-immunity studies between the two related genotypes found in *T. parva* Katete revealed that there was no important immunological difference between the two genotypes. However, further bovine passages changed the dominance in that the less dominant genotype became the prominent genotype from the second passage onwards. A similar result was found in a characterisation study of parasites circulating after a heterologous challenge in an experimental animal. Only the challenge parasite genotype could be picked up by PCR amplification (own results). The nature of dominance might be different in the two instances but the biological relevance of a dominant genotype in relation to the immune response is not entirely clear.

Gupta et al. (1996) argued that competition among different components present in a parasite stock would lead to the exclusion of subpopulations carrying similar dominant epitopes. In the case of the Zambian isolates, it could be argued that immune pressure caused the dominant, probably sole genotype present, to evolve. Immune pressure could be reflected in changes in sequences. The shorter PIM sequences derived from an existing longer form might be indicative of such pressure and the fact that identical changes have been detected in the field, could be an indication of a superior fitness of these shorter PIM forms. Further evidence that the predominant genotype is evolving can be found in the presence of similar genotypes in the field in Eastern Province after immunisation with *T. parva* Katete. In the case of the complex *T. parva* Marikebuni stock, which includes a Muguga type PIM gene, it has been shown that *T. parva* Marikebuni provides complete protection against challenge

with *T. parva* Muguga but reciprocal protection is not provided. It can be argued that at least two subpopulations of parasites, with different immuno-dominant epitopes, make up the Marikebuni stock. The Muguga-like component provides the protection against a Muguga challenge but a different and dominant PIM genotype in Marikebuni causes breakthroughs in Muguga immunised animals. The alternative explanation, that different epitopes present in the dominant Marikebuni genotype are responsible for the protection to *T. parva* Muguga challenge and breakthrough, would be contradicted by the cross-immunity results. This confirms the multi-component nature of certain *T. parva* stocks but also highlights how dynamic it is. It follows that cross-protection characteristics of such stocks would be of an ephemeral nature. If proven right, this would have serious repercussions on current vaccination procedures in which the use of a complex stock is often preferred in order to obtain as large an array as possible of different parasite populations, and provide a wide immunogenic cover.

If a vaccine stock consists of genotypes in different proportions, the loss of one genotype might occur during stabilate preparation and might jeopardise its protection efficiency. Only expensive cross-immunity trials of each vaccine batch would indicate the loss of an important component and bar it for field immunisation. This provides another strong argument for the use of molecular based techniques for the characterisation of vaccine stocks and of each vaccine batch. Allelic marker assays would be ideal in view of their clear and direct information. Some important but minor genotype might be revealed only after cloning of the parasite stock. It is obvious from the above discussion that a single component vaccine has an important advantage in this respect, providing a stable product. However, a single component vaccine providing the broadly protection needed in the eastern African situation might be difficult to find.

The results of the survey of southern Zambian parasites show the presence of a predominant genotype over a wide area of the province that causes high mortality. Such a situation consists of a parasite population that has been defined as having an epidemic structure created through the selection and subsequent spread of a particular allelic genotype that becomes the dominant parasite genotype. It is difficult to explain the spread of the newly introduced

stock under any other population structure. The local stocks have been shown to provide full protection against the components of the cocktail vaccine (Musisi *et al.*, 1986; Asveza South cross-immunity data). It is difficult to conceive of the expansion of a cocktail component genotype under an endemic situation in a cattle population showing high levels of cross-immunity.

An alternative hypothesis is that the Mazabuka stock is a recombinant parasite with different immunogenic properties, causing breakthrough infections in animals carrying the wild strains. There are some indications that this could be the case; there are only small genome differences and mortality due to ECF has been confirmed recently in some cocktail immunised animals challenged with Mazabuka (Asveza South results). However consistent proof has not been found. Confirmation of this hypothesis depends on the production of a *T. parva* Mazabuka stabilate from the existing tissue culture isolate in order to characterise its cross-protection profile.

A third but less plausible hypothesis is that a similar genotype to one of the cocktail components was present before the introduction of the cocktail. This is based on the characterisation of a similar genotype (P. Spooner, personal communication) isolated at the lake shore in Kasungu District of Malawi in 1977 (K. Kanhai, personal communication; FAO project report). The fact that from 1980 onwards, numerous isolates have been characterised by mAbs in Malawi (FAO project) and Zambia (BADC project, Chipata and FAO project, Balmoral) and did not reveal any other isolate with a Muguga type mAb profile renders this hypothesis unlikely. Moreover, mAbs profiles are related to small regions in the PIM sequence of a *T. parva* parasite and cannot be used to predict sequence similarity as shown in the case of the PIM results of the Zam5 isolate (see under 4.4.2). The profiles of the three allelic markers are sufficiently different for the Zambian and exotic stocks, and for the Kiambu5 and the Muguga and Serengeti-transformed stocks, to reveal mixed infections if present. The low numbers of mixed infections in Zambia would facilitate the tracking of recombinants as the presence of multiple infections in field samples is considered as a major stumbling block in characterisation of the various genotypes making up the mixture (Paul and Day, 1998).

The stability of the allelic frequencies in the field population is another important factor in relation to the usefulness of characterisation methods. The population dynamics are correlated with the epidemiological character of the disease in a certain region. It can be predicted from *Plasmodium* survey results from a meso-endemic area (50% of the samples showing multiple genotypes) that population dynamics need to be assessed over longer periods (Ferreira *et al.*, 1998). It has been reported in *P. falciparum* studies that patterns of allelic diversity (19 different alleles) at the MSP-1 locus in 102 clinical isolates remained stable over a two year period (Ferreira *et al.*, 1998). A similar stability in allelic frequencies in an hyper-endemic area in the Gambia (average of two genotypes per sample) over a seven year period was reported by Conway *et al.*, (1992). These authors examined for frequency-dependent immune selection mechanisms in maintaining this antigenic polymorphism but found none. They concluded that frequencies of MSP-1 alleles are probably not maintained by immune selection. Kyes *et al.* (1997) reported similar results from a longitudinal study in an endemic area (average of two genotypes per sample) in Kenya using MSP-1 and MSP-2 allelic marker assays. Ferreira *et al.* (1998) came to the same conclusion after comparing allelic diversity at the MSP-1 locus in geographically different locations with different endemicity. In contrast, year to year variation in specificity of serological responses to PfEMP1 var gene epitopes was reported from Sudan (Giha, 1997), whereas anti-var antibodies were found to be stable over a three year period in Papua New Guinea (Piper, 1997). These differences could be explained by a lower transmission intensity in Papua New Guinea, although the reliability of the anti-var detection assays could be questioned.

It can be concluded from field studies of *Plasmodium* that some evidence has been found to support the hypothesis that var genes might structure field populations, although better characterisation assays are needed. The promising results obtained with flow cytometry in studying var gene expression might remedy this (Staalsoe, 1997; Hayward, 1997).

4.4. Analysis of PIM sequences

4.4.1. PIM nucleotide sequences

The unique nature of the PIM gene is supported by the absence of related sequences in database search of other pathogens, as reported by Baylis *et al.* (1993). PIM has been shown to be a single copy gene and allelic differences are encoded by individual parasites with different genotypes (Toye *et al.,* 1995). It has typical characteristics of a membrane protein with a potential signal sequence at the N-terminal end, a membrane spanning domain near the C-terminus followed by a short hydrophilic tail (Baylis *et al.,* 1993). Three potential N-glycosylation sites have been identified but no polymorphism was found in adjacent sequences, indicating that these are not involved in immune responses. The PIM gene structure is different from what is known of the var gene sequences in *P. falciparum* (Kyes *et al.,* 1997). In the latter, point mutations within conserved blocks forming two to five Duffy-binding-like domains can be recognised in an otherwise highly variable first exon. There are also obvious differences between the polymorphism found in PIM and the well studied MSP-1 gene. In the latter, a stable dimorphism is found although high numbers of different alleles have been found that are generated through intra-genic recombination.

There is a large area of repeated short sequences in most bovine *T. parva* isolates that is absent from *T. parva* Katete, *T. lawrencei* and buffalo-derived isolates. Pepscan analysis with bovine antiserum confirmed the presence of dominant B-cell epitopes within these PIM repeat domains (Toye *et al.,* 1996). Various hypotheses have been proposed to explain the high frequency of similar, short tandem repeated sequences in polymorphic *Plasmodium* antigens. The main theme revolves around the functional relationship of these repeats and uses the observations of the conserved nature of its sequence polymorphism together with the universal presence of these repeats in *Plasmodium* as main arguments. The most accepted theories are those that attribute immune interference properties to the repeats. The function of

smokescreen antigens (Anders *et al.,* 1983; Kemp *et al.,* 1987) is to divert immune responses away from antigens necessary for parasite survival. The prevention of high affinity antibody responses by the presence of variable, but cross-reacting epitopes, is another possibility (Anders, 1986; Kemp *et al.,* 1987). The repeats might mimic host protein epitopes thereby avoiding immune recognition as suggested by Kobayashi *et al.* (1986) and McLaughlin *et al.* (1987). Simple amino acid polymers elicit T-cell independent antibody responses. Enea and Arnot (1988) argue that the role of repeats in parasite antigens is to elicit just such a response which is relatively inefficient in eliminating infections and does not confer lasting immunity.

These repeats could also attract or divert the dominant interactions from adjacent epitopes towards more distal epitopes, thereby interfering with the protective responses, in either a negative or positive way. Centrally positioned, short tandemly repeated sequences in the *Plasmodium* SERA antigen have been shown to be the major antibody binding sites (Kemp *et al.,* 1987) that are undergoing dramatic change in sequence, length and number of repeats. It could be argued that conserved epitopes in the PIM gene might play a dominant role in protective responses but that the high polymorphism in the central region of PIM aids in diverting an effective immune response away from these epitopes. Although these repeats are well conserved when present, the fact that they can be absent in some field stocks does not support a correlation with the traditional concepts of interference and immune evasion. This concept was challenged by Frontali and Pizzi (1991) after studying tandemly repeated families among *Plasmodium* species. They concluded that the presence of identical repeats in geographically distant strains might not imply a conserved biological role but might point to a common ancestry. It is striking that the tandem repeats among *T. parva* strains are identical in sequence (PQPE) while *T. lawrencei* has a mixture of two different repeats (PEPE and PQPQ) and buffalo-derived *T. parva* a mixture of *T. lawrencei* (PEPE/PQPQ) and *T. parva* (PQPE) sequences (Table 19). Although more sequences from buffalo need to be compared, it is tempting to associate this with phylogenetic evidence for the longterm divergence between *T. lawrencei* and *T. parva* parasites, and the intermediate status of

isolates such as *T. parva* Boleni and Zam5. This also agrees with the p67 data for these strains.

The analysis of PIM gene structures from different isolates reveals a general design characterised by multiple domains that are highly conserved and are shared among several isolates. Other blocks appear either to be common among stocks from the same geographical region or to be stock-specific. This gives rise to a mosaic appearance of the gene due to the alternation of shared blocks, deletions and specific sequences The shared sequences might have resulted from common ancestry (McDevitt, 1995), recombination or gene conversion (Parham *et al.,* 1995) or convergent evolution (Klein and O'hUigin, 1995). Immunoglobulin gene regions also consist of a patchwork pattern of sequence motifs and the underlying mechanisms leading to the generation of the mosaic structure have been discussed by several authors (Lawlor *et al.,* 1990; Gyllensten *et al.,* 1991; Parham *et al.,* 1995). Intra-locus recombination or gene conversion can generate a mosaic appearance but only when there is a pre-existing polymorphism in the population. This implies that balancing selection would ultimately be responsible for the sequence motifs (Satta, 1997). Both mechanisms could be incriminated in the generation of the mosaic appearance of the PIM gene as sufficient regions of homology are present to initiate intragenic recombination events. On the other hand, several X-like recombination motifs (Table 23) could be identified and might be responsible for initiation of gene conversion events (see under section 1.3.3.3.) although an identical motif was not present. Strikingly similar motifs have been identified also in other protozoan parasites such as *Leishmania* spp (Liu *et al.,* 1992) and *Trypanosoma brucei* (Le Blancq *et al.,* 1988) and were incriminated in reciprocal cross-over events.

x-like motifs in *T. parva*				
GCTGGGG	GGGTGATTC	GTGGGG	CAGGGGGAT	CCCGTAG
GCTGGTG	GGGTG	GATGGGC	CAGGGTGAT	CCTGTAT
CCTGTGG	GTGGAC	CTCCGG	GCCTCGGCCTC	
GGTCAGG		GATGGTC	GACTCCTGATG	
x- motifs from literature				
GCTGGTGG				
GGAGGTAGGCAGGCAG				
GTGACTGGCCAGGAGG				
GGGCAGGAXG				
CXTCCTGCCC				

Table 23. X-like recombination motifs found in all PIM sequences

Gene conversion seems a more acceptable mechanism for explaining the polymorphism than intragenic recombination, as the latter would imply an implausible frequency of recombination. Gene conversion and reciprocal intergenic exchanges have been identified as mechanisms responsible for the re-shuffling of important epitopes, thereby creating novel alleles (Dormoy *et al.,* 1997).

The fact that three sequences reveal large deletions which could be related to the loss of a large continuous part of sequence, as in the case of the Zambian stocks, is remarkable. The immunological repercussion of PIM size on cross protection was assessed using two Katete isolates with different PIM alleles. The Katete stock with the small sized PIM showed full protection against the original Katete stock carrying the long sized PIM. The Katete short sequence has been found to be dominant in the parasite used for vaccination and is the sequence presently found in the field. This may be proof of its superior fitness but its wide distribution may also be the result of the years of vaccination.

A second important finding is the relationship among buffalo-derived *T. parva* sequences and *T. lawrencei*. *Theileria parva* Boleni and Zam5 display the highest sequence homology with *T. lawrencei* as can be seen from Fig.35. This agrees with results reported by Toye *et al.* (1995) but extends them to the entire variable area of the gene and to more stocks. The size and sequences of the intron near the 3' end is identical (except for two changes in the Boleni

sequence) for all PIM genes so far analysed and is in marked contrast to the polymorphism found in the coding part of the gene. This suggests that the sequences in the coding regions are under selective pressure most likely imposed by the immune system of the host.

Polymorphism in genes can be the result of basicaly three forces: immune selection pressure, genetic drift or divergent directional change (Conway, 1997). Extensive polymorphism in surface proteins of parasites is a strong indication that immune selection pressure forces are at work. It has been shown that PIM is a predominant schizont antigen with reactivity to sera from infected animals as shown on immunoblots (Toye *et al.*, 1991). The mosaic nature of the polymorphism found in PIM excludes genetic drift or directional change as possible forces. Extensive polymorphism was also found in various *Plasmodium sp* genes coding for surface antigens, but no correlation was found with protective antibodies.

An indication that the PIM protein might induce neutralising antibodies was given by Toye *et al.* (1996) who reported the inhibition of sporozoite infectivity *in vitro* by anti-PIM Mab (Toye *et al.*, 1995). More importantly, a study ot the presence of CTL epitopes in the PIM sequence need to be carried out as CTL-based cellular immunity has been shown to be the main mechanism of protection against *T. parva* infection (McKeever *et al.*, 1994; Taracha *et al.*, 1995).

4.4.2. PIM sequence-derived amino acids

Many specific sequence blocks are shared among *T. lawrencei* and *T. parva* Boleni and Zam5. These are unique for the three stocks and can be used to differentiate them from the remaining sequences as buffalo-like sequences. *Theileria parva* Mazabuka is the only cattle isolate that shares a nonapeptide with *T. lawrencei* and Zam5. This sequence PVD(Q/H)QQ(Q)P(V/T) is imperfectly repeated five times (*T. lawrencei*), four times (Zam5) and one time (*T. parva* Boleni) in the buffalo-like sequences. Block A and Q are remarkably conserved in cattle stocks whereas they contain related variable sequences and point mutations in the buffalo-like sequences. Sequence analysis indicates the presence of hydrophobic regions. The C-terminal has a predicted hydrophobic

region and suggests that this region might function as an anchor of the polypeptide in the parasite membrane (Kawazu *et al.*, 1992) and would explain its conserved nature. The N-terminal region has a putative signal sequence showing a remarkable conservation. This conservation is not always functionally determined as these regions in VSP genes of *Giardia* have been reported to show polymorphism (Nash *et al.*, 1995).

The PIM epitopes recognised by mAbs 2-7, IL-S32.3 and IL-S34.3 have been determined (Toye *et al.*, 1996) and the sequence data correlate well with the mAbs results, and with the predicted presence of the epitopes (Table 24). Although the sequence of Zam5 differs to a large extent from the other Zambian isolates, its mAbs profile is identical. This could be explained by the presence of a very similar peptide, differing only in the middle amino acid (DQP**V**DHQ) position. The location of this difference would probably not restrict antibody interaction and therefore not affect its reactivity with mAb7. This shows the limited value of the mAb assay as a characterisation tool. The RFLP-PCR profiles show that Zam5 differs markedly from the other Zambian isolates.

Table 24. PIM sequences of epitopes recognised by the different mAbs.

mAb origin	mAbs	peptide epitopes	peptide epitopes	peptide epitopes	Zams	Mug	comments
Muguga	2	HQPTPAA			0	3	
Marikebuni	7	DQPDQHQ	PVYQQQPVQQPS		1	3	All stocks except buff. derived
Marikebuni	**15**				0	0	
Marikebuni	IL-S32.3	**ASGEVPVKPSEG**			0	0	All stocks except lawrencei
	IL-S34.3	DQPDQHQ	PVYQQQP	QPDGHHQ	0	1	

It is not possible to comment on the mechanisms of conservation or evolution of the sequences making up the repeat region as the sequence data for these areas are less reliable. The method used to obtain the PIM sequences was based on a single run. The method consists of a single strand reading starting from the terminal ends of the amplified product and continuing for about 500 bp. The reading becomes unreliable from bp 400 in both directions, coinciding with the repeated sequence region in the long sized PIM products.

4.5. Origin of Zambian stocks

The results of Southern blot profiles suggest a common origin for the Zambian stocks, except for the Zam5 isolate. The allelic markers are different only for the PIM gene while the sequence comparison of the polymorphic region reveals a high homology among the Zambian stocks when analysing the sequences in matrix plots (results not shown). The Zam5 sequence shares identical (thirteen) or similar (four and three) peptides with the Zambian South isolates and a similar six-mer peptide with all Zambian isolates (Fig. 38). It shares a unique tetrapeptide with the Boleni sequence. There is not a single common sequence block between the Boleni and the other Zambian isolates, but several sequence blocks are similar to Muguga (ten peptides shared with all Zambian isolates and three with the Southern isolates) or Marikebuni (18 shared with all and ten with the Eastern isolates). These data all point towards a northern descent of the Zambian isolates with the exception of Zam5. The allelic results on the other genes, coupled with the PIM sequence analysis, suggest a closer relationship of the Zambian isolates with the *T. parva* Marikebuni isolate. The Zam5 stock shows all the characteristics of a buffalo-like sequence and the fact that it has not been possible in four attempts to infect an experimental bovine with the cell line supports the buffalo-type origin of this stock although other reasons for this failure cannot be ruled out. There is a closer homology between the Boleni and *T. lawrencei* stock than with Zam5.

4.6. Significance of the PIM gene as a marker

The definition of random mating implies that the characteristics of a particular strain in a parasite population exhibiting random mating are ephemeral. On the other hand, it has been argued that various mechanisms would restrict this random mating behaviour, leading to a division of the population into subpopulations. The longevity of these subpopulations depends on the degree of restriction interfering with their random mating behaviour. The various mechanisms structuring parasite populations into subpopulations can be considered as the basis for the strain concept (Gupta and Day, 1994). The most

obvious mechanism influencing strain diversity for a pathogen like *T. parva* in the Zambian situation would be immunologically based. The definition of a strain under these conditions is a distinct population of parasites determined by the common immune response they elicit in the host thereby interfering with infection and transmission of their homologues. This implies that strains are characterised by differences in immuno-dominant, protective proteins. Co-existence of different strains in the same host would mean that there is no cross-protection among the strains present. This would structure the parasite population into different strains. The extensive cross-immunity differences found in *Theileria*, particularly in *T. parva*, could be explained in this way.

In a situation characterised by the existence of many strains it would be critical for their longevity that the immune response is targeted against a range of parasite proteins, each contributing only partly to the protective response. If this is the case in *T. parva*, the variation in challenge reactions would be much larger than what is usually seen in cross-immunity data, as many combinations among the different antigens could and would be formed in field populations. Immunity after infection would not be solid unless the host has been in contact with most of the wild strains present in the area, as argued in the case of *Plasmodium* infections by Gupta and Day (1994) and Young *et al.* (1987) for *T. parva* in an endemic area.

In view of the solid immunity engendered by natural *T. parva* infections in different epidemiological environments, the situation will be different and might be characterised by the presence of a few different strains in a defined area. These would exhibit a limited epitope range that might be partly or, exceptionally, totally different among the various strains. The immunity would be characterised by an immuno-dominant response dependant on the hierarchy and interaction amongst the epitopes.

The limited strain hypothesis looks the most likely in view of the data presented and suggests that the diversity could be limited to one or a few genes, coding for a highly immunogenic protein. The structure of field populations would also

imply that it would be possible to identify the gene(s) encoding immunogenic proteins. In the case of *T. parva*, the results from the RFLP-PCR characterisation indicate that PIM is the most polymorphic antigen, dividing the parasite populations between Eastern and Southern Province, as well as two (Muguga and Kiambu 5) of the cocktail vaccine components. The characterisation data obtained for the Muguga and Serengeti components indicate a close identity between the two stocks. The results obtained from exotic stocks with the PIM marker underscore the hypothesis on the role of this antigen. Profiles from the Marikebuni stocks and a clone, showing distinct genotypes, including one identical to Muguga, correlate well with the cross-immunity results reported under section 1.1.5.2. Moreover, results from the characterisation of field samples using the PIM marker reveal a stable dominant profile, identical to one of the vaccine components, used more than five years ago. This provides strong evidence for the usefulness of this marker in characterising different parasite populations in the field.

The Tpr1 probe and the mAb profiles for Marikebuni were different from those of Muguga (Conrad *et al.,* 1989; Toye *et al.,* 1990). Morrison *et al.* (1987) showed that differences in CTL responses elicited by animals immunised with one of the above mentioned strains correlated with protective immunity. The different cross-immunity profiles also correlate well with the sequence data of the PIM gene obtained from different but limited number of stocks. Sequence data from PIM genes from other stocks need to be obtained in order gain more information on the gene structure and its possible importance in structuring the parasite populations.

Various hypotheses could be formulated about the role of the PIM gene in relation to host immune responses.

1. Antibodies elicited by this immunodominant antigen lack any immunoprotective effect (A. Musoke and S. Morzaria, personal communication). PIM might be a surface antigen eliciting B cell responses without protection, overloading the host immune system and diverting the reaction from the real protective epitopes (smoke screen antigens). This has been suggested for antigens expressed on the surface of erythrocytes infected with the blood stage

of *Plasmodium*. It would have no evolutionary advantage for a parasite residing in a lymphocyte where the initiation of cell mediated immune responses have been shown to play the important role in its control. Moreover, there is good evidence that antibodies do not protect against *T. parva* infections at the schizont stage and it would be difficult to explain how the present extensive polymorphism would be maintained.

2. PIM might be one of the many polymorphic antigen genes contributing towards an efficient immune response (polygenic immune response). In such a case, immunity would probably be built up gradually, needing exposure to different epitopes. Field data seem to contradict this, as a usually broad and solid immunity is acquired after one exposure. Although CTL responses have been shown to be important in strain specific immunity, there is evidence that other mechanisms might be involved in protective immunity against *T. parva*. But these might be less specific in nature and hence only have a helper role.

3. PIM might be linked to a dominant antigen. This seems unlikely because of the correlation between PIM sequence and cross-immunity data, albeit from a limited number of stocks. This would still imply that PIM might be an important marker for strain characterisation.

4. PIM is the immunodominant antigen, determining protective immune responses. Further research should be directed to obtaining more sequence data from stocks for which good cross-immunity data are available. Comparison of sequence data would shed light on possible protective epitopes and add to the understanding of the PIM gene structure, and the mechanisms underlying diversity.

4.7. Phylogenetic position

Analysis of the characterisation data from this study has given new insights and prompted a review of the specific position of the different parasite types in the *T. parva* complex. The role of buffalo-derived parasites in the epidemiology of *T. parva* is still far from clear but areas for further research have been identified. The specific position of *T. lawrencei* in relation to *T. parva* can be questioned in the light of increasing molecular data identifying genetic differences. The

analysis of limited but geographically widespread samples reveals the existence of two types of *T. parva* isolates (Fig. 53). One, a cattle-derived parasite showing dimorphism in the antigen genes of p104, p32 and p150 and no polymorphism in p67. This limited polymorphism is in sharp contrast to the polymorphism found in the buffalo-derived parasite, for the same genes. Analysis of polymorphism of ribosomal genes and ITS regions of *T. parva* (N=28) and *T. lawrencei* (N=22) isolates failed to reveal species-conserved sequences that would be useful for differentiation. Many buffalo-derived parasites showed a mosaic pattern in the *T. lawrencei* and *T. parva* -derived sequences in the ITS region, whereas the ribosomal genes were identical (Collins, 1997).

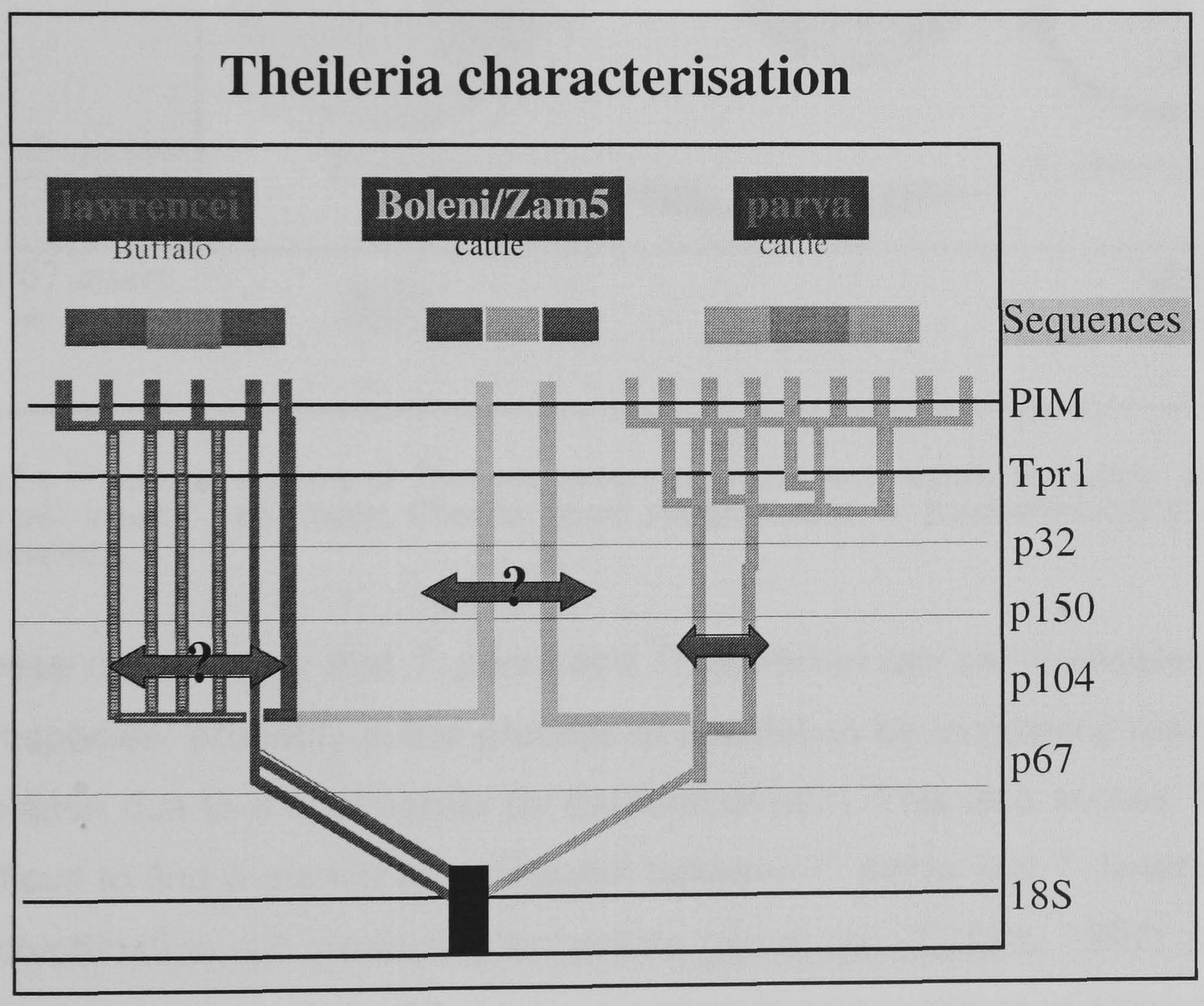

Fig. 53. Diagram of characterisation results of *T. parva*, *T. lawrencei* and buffalo-derived *T. parva* stocks with at the righthand side the various loci. Sequences are derived from the PIM locus.

A similar picture appeared after alignment of p67 sequences, showing extensive polymorphism and a >100bp insert in *T. lawrencei* parasites. *Theileria parva* parasites did not show any polymorphism and lacked the insert. Only two exceptions (one Zimbabwean and one South African isolate) were found in

which a *T. lawrencei* profile did not have an insert (Collins, 1997). These data support the occurrence of recombination between *T. parva* and *T. lawrencei*. This finding is in line with results obtained in the present study on the PIM gene, showing recombination between *T. parva* and *T. lawrencei*.

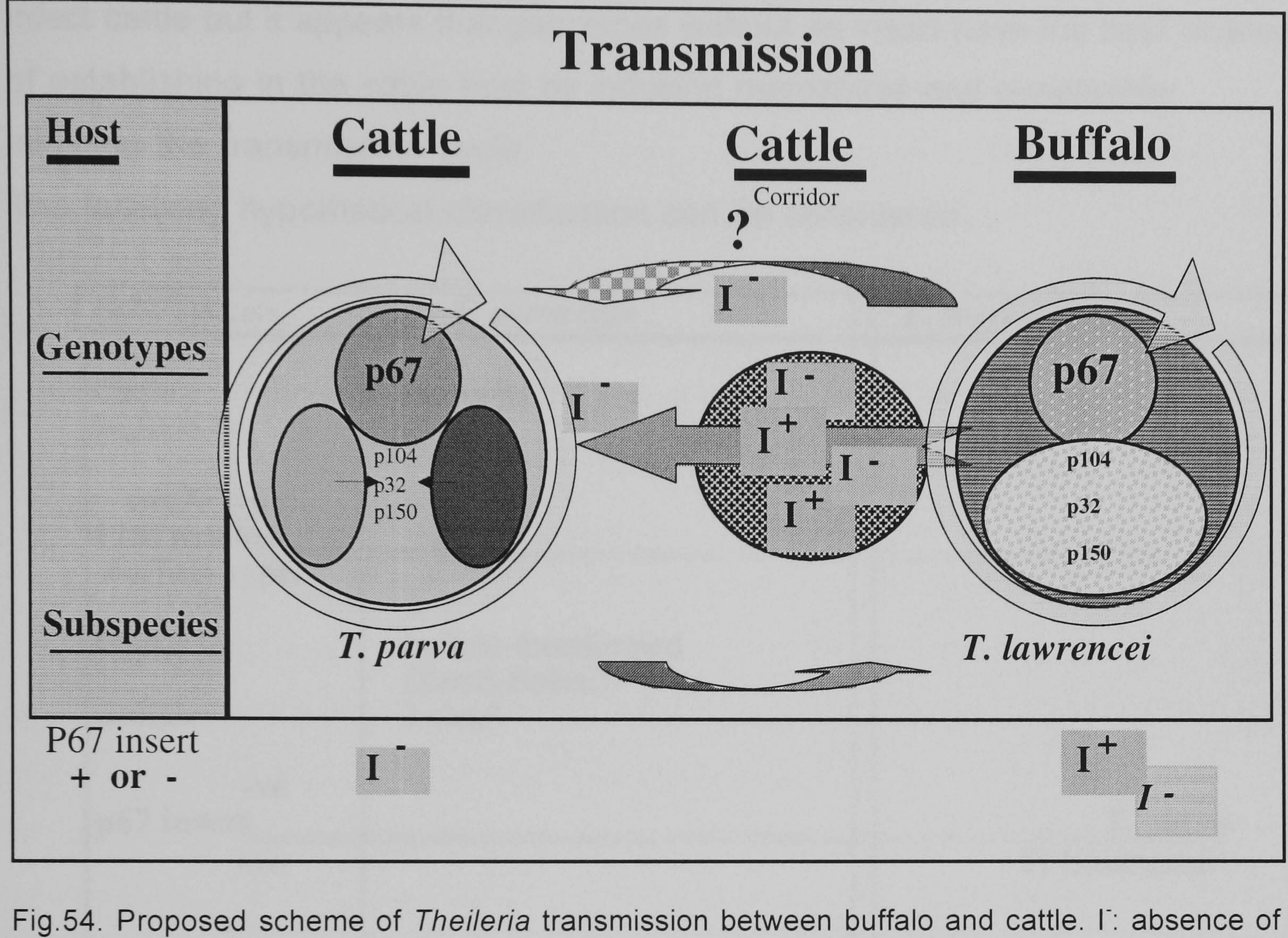

Fig.54. Proposed scheme of *Theileria* transmission between buffalo and cattle. I^-: absence of the p67 insert. I^+: p67 insert. Checker board patterns stand for polymorphisms of markers indicated.

These results imply that *T. parva* and *T. lawrencei* can be considered as subspecies, probably in the process of speciation by increasing reproductive isolation due to a host barrier (in the form of p67). This also implies that it will be difficult to find a marker to distinguish between *T. parva* and *T. lawrencei* as recombination will produce intermediate genotypes (Collins, 1997). It is tempting to argue that p67 plays an important role in the speciation process by increasing the fitness of the parasite for the bovine host as the majority of cattle-derived *T. parva* parasites have a specific and extremely conserved p67 sequence. The data are consistent with the establishment of a subpopulation of *T. lawrencei* parasites in the bovine host (Fig. 54). The infectivity of *T. lawrencei* parasites for cattle is not p67 dependant, but p67 seems to be associated with the successful establishment in the cattle host. Data from South Africa show that

parasites without a p67 insert can cycle in buffalo and compete with *T. lawrencei*. Recombination can occur either between such resident parasites, without a p67 insert, and *T. lawrencei* parasites, or between *T. parva* and *T. lawrencei* parasites. *Theileria lawrencei* or recombinant parasites have been shown to infect cattle but it appears that genotypes without an insert have the best chance of establishing in the cattle host by inducing merozoites and piroplasms, initiating the transmission cycle.

The following hypothetical classification can be considered.

Host/Species	***T. parva type***	***T. lawrencei type.***
Cattle Buffalo	**majority** *likely?*[1]	N/A *not known?*[3]
cattle type PIM marker **buffalo type**		
Cattle Buffalo	buffalo-transformed (Zam5-Boleni) 1 case[4]	
-ve **p67 insert** **+ve**		**T. parva** **T. Lawrencei**
cattle Buffalo	buffalo-derived[2] (Corridor)?	corridor **majority**
18S probe polymorphism		

Table 25. Proposed classification for *T. parva* and *T. lawrencei* distinction, and the relevant loci. Words in italics indicate hypothetical genotypes awaiting characterisation. N/A: not applicable.

1. Not known but very likely to exist (Barnett and Brocklesby, 1966).
2. One case found, but no data on p32 and PIM sequences.
3. Not known but likely to exist.
4. Data from Collins, 1996. Could also be a *T. parva* in buffalo as p32 and PIM sequences are not known.

The results on the p67 analysis suggest that the presence of an insert is unique for *T. lawrencei* or buffalo-derived stocks, and does not prevent infection in cattle. It is present in the majority of buffalo-derived isolates.

The fact that a p67 insert is found in the first passage of an isolate of what could be best defined as a *T. lawrencei* stock, Boleni, but lost during subsequent passages, and absent from Zam5, another stock with buffalo-derived

characteristics, is intriguing. The absence of the insert seems to be correlated with efficient transmissibility. Whether this is related to changes in the multiplication rate, increased rates of merogony or increased invasion of red blood cells is not known. The presence of a p67 insert in a readily transmissable isolate has not been demonstrated. If buffalo-derived parasites are transmitted to cattle, transmission in the opposite direction would also be possible and cattle parasites will become established in the buffalo as has been reported by Barnett and Brocklesby (1966) and Grootenhuis *et al.* (1987). It is possible that these *T. parva* parasites might recombine in the tick, giving rise to mixed genotypes with the p67 genotype of a *T. parva* parasite, and a PIM genotype of the *T. lawrencei* parasite, resulting in a cattle-derived *T. lawrencei* type. The sequence data of the PIM gene suggest important areas of difference between *T. parva* on the one hand and *T. lawrencei* and buffalo-derived stocks on the other. It has been shown recently that recombination occurs in the field between *T. parva* and *T. lawrencei* and that there is no molecular basis for a species distinction (Collins, 1997). Two possibilities might be considered in explaining the p67 results. It might be that a subpopulation, lacking the p67 insert, exists in some buffalo and is selected when introduced in cattle but competition with fitter wild type parasites will prevent its establishment in the cattle population. It is more likely that cattle-type *T. parva* parasites infect buffalo and, after recombination, might be re-introduced to cattle. But the establishment of recombinant genotypes will be difficult as most will have a lower fitness compared to the wild type(s). The p67 results, the p104 and p32 profiles and the analysis of repeated sequences in the PIM gene suggest the existence of three groups which could be classified as (i) buffalo-derived *T. parva* (p67 insert positive), (ii) buffalo-type *T. parva* and (iii) cattle-type *T. parva* (p67 insert negative). The p32 and p104 loci could be important in differentiating buffalo-type parasites from cattle parasites, but data on buffalo-derived stocks in relation to these loci are lacking. In the light of the previous argument it can be anticipated that the relationship between *T. lawrencei* and *T. parva* is likely to be complex. The fact that a Zimbabwe survey revealed different Tpr1 profiles in cattle and buffalo-derived parasites (Bishop *et al.,* 1994) and the inconclusive results from the transformation studies (reviewed in this thesis) might indicate important

biological differences between the parasites. Another important factor already mentioned could be the influence of the different tick vectors. The importance of buffalo-derived infections in the *T. parva* epidemiology might be influenced also by the presence of *R. zambesiensis* that is reported to be a better vector of *T. lawrencei* (Lawrence, 1983; Blouin and Stoltsz, 1989). The p67 and Tpr1 results from Southern Africa question the frequency of transformation from buffalo to cattle-adapted parasites, and the Boleni stock might turn out to be an important isolate in this respect. It is felt that the molecular tools used in this characterisation study could form a sound basis for investigating the *T. parva* parasite populations in cattle areas with close buffalo contact and assist in determining the frequency of transformation.

It is well recognised that the presently accepted nomenclature and classification of *Theileria* can only be changed when substantial data are available that justify it. Nonetheless, the present nomenclature is confusing and may confound the efficient communication of results. Adoption of a dual nomenclature system based on host (buffalo-derived *T. parva* and *T. parva*) and type (buffalo- and cattle-type) would improve the clarity of reporting.

5. Conclusion

Molecular based analysis of parasites has contributed substantially to our understanding of parasite relationships (phylogeny), parasite population structures (molecular genetics) and parasite-host-vector relationships (epidemiology). This study highlights the field application of PCR-based assays using highly polymorphic genes and shows the important advantages of such an approach in the study of *T. parva* parasites.

1. The *in vitro* studies on the stability of the *T. parva* genome over time have demonstrated the usefulness of the probes and markers selected in characterising parasite stocks and clones. Three of the probes used (Telomere, Minisat and LA6) have the advantage of informing on polymorphisms in sequences spread over the entire genome. The results obtained with the probes correlated well with the results of the markers. The cloning of parasite isolates indicated homogeneity of the parasite populations although selection during *in*

vitro culture could not be ruled out. It also showed the practical superiority of the markers compared to the probe results. It was shown that the Tpr1 probe profiles are evolving rapidly and very careful interpretation of these results is required.

2. The extraction of blood spots on filter paper and PCR assays proved very robust and practical for field samples. The fact that more than 90% of extracts from field samples were found positive using the 18S assay demonstrates the usefulness of the method. PCR amplification of blood from field samples will not always yield a result due to fluctuations of the parasitaemia in carrier animals. Preliminary data from quantification studies indicated that differences in fluctuation are substantial and suggest that only 50% of the samples from carrier animals might be positive. It can be concluded that the characterisation of field populations is feasable using the assays based on the three markers (p104, p150 and PIM).

3. The study has also shown the feasability of determining the field population structure in the Southern Province of Zambia.

The influence of epidemiological parameters on the parasite population structure was shown and the outcome of the introduction of exotic parasites revealed. But it is uncertain if the same set of markers will be capable of providing sufficient resolution under a different epidemiological situation in which high recombination rates prevail. It is important to find additional markers, preferably on chromosomes one and two, in order to substanciate the information obtained from genotyping field populates.

4. The study showed that the characterisation tools that have been developed can be used to examine the composition and dynamics of field populations. Characterisation results have been used with success to reveal relationships between genotypes and identify geographical related genotypes. The multi-locus characterisation profiles of the recent field isolates from the Southern Province are identical to the Mazabuka genotype which could be related to the genotype of two of the components of the Muguga cocktail. The parasite populations showed homogeneity under Zambian epidemiological conditions, before the introduction of exotic stocks. Thereafter, homogeneity was found in an extensive area of the Southern Province but a parasite with a genotype similar to one of the

components of the immunising combination of stocks became dominant. It could not be determined if recombination had occurred between the introduced and local parasites, although no recombinants were identified in the small number of field samples examined. The rapid spread of the introduced genotype can be explained by the specific epidemiological situation of *T. parva* found in the Southern Province.

Information on the dynamics of the carrier state in *T. parva* epidemiology have been presented which warrant further investigation in order to qualify and quantify its role under different epidemiological conditions.

Data of PIM sequences suggest an important role for this gene in the immune response to *T. parva*. It is an immuno-dominant antigen expressed by an intracellular stage of the parasite and located in the parasite membrane, as shown by IFAT staining. Most monoclonal antibodies raised against this parasite stage recognise PIM and detect antigenic diversity in *T. parva*. Although CTL epitopes have not been identified, the characterisation of PIM sequences of field parasites reported here suggests a correlation between this antigen and a structuring of the field population. This is indicative of a potential importance for this antigen in the generation of protective immune responses. An alternative hypothesis would be a linkage of this gene with an important antigen. The sequence data from different but a limited number of stocks correlate with cross-immunity data. Further research is needed to confirm these findings and could be important, not only to the understanding of the structure and underlying diversity mechanism affecting this gene, but also in the search for a less demanding identification method of important protective epitopes.

The analysis of the characterisation data in this study has shown a dimorphism in cattle-derived parasites. Data have been provided which are consistent with a speciation process whereby a subpopulation of *T. lawrencei* parasites have established themselves in the cattle host. A proposal of a dual nomenclature system based on host (buffalo-derived *T. parva* and *T. parva*) and type (buffalo- and cattle-type) was made to improve the clarity of reporting.

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