

it is likely that modern molecular techniques will resolve the issue.

HpaII endonuclease distinguishes between two species in the *Anopheles funestus* group

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Abstract

The *Anopheles funestus* group consists of at least eight species that are currently identified mainly on morphological criteria. Until recently, only *An. funestus* s.s. was implicated in the transmission of malaria in Africa, but recent work in Tanzania has shown that *An. rivulorum* is also involved, albeit to a lesser degree than *An. funestus*. The constraints in the identification of the species and the need to clarify better their epidemiological role have led to the development of a PCR-RFLP method for the identification of two anthropophilic members of the group. Using PCR primers developed from the D3 region in the 28S ribosomal gene, amplified products were digested with the restriction endonuclease HpaII. This produced two distinct fragments on an agarose gel that could be used to separate *An. funestus* from *An. vaneedeni*. The technique needs to be tested on natural populations of these two species as well as on other members of the *An. funestus* group.

Keywords: *Anopheles funestus*, DNA identification, restriction enzyme endonuclease.

Introduction

Anopheles funestus Giles is widely distributed throughout tropical Africa and is one of the three major vectors of human malaria parasites on the continent. It belongs to a group of at least eight species that are difficult or impossible to distinguish morphologically in the adult stage (see Gillies & De Meillon, 1968; Gillies & Coetzee, 1987). *Anopheles*

rivulorum Leeson, also widespread, is the only other member of the group that has been implicated in malaria transmission, that being very recently in field-based studies in Tanzania (Wilkes *et al.*, 1996). *Anopheles vaneedeni* Gillies & Coetzee was experimentally infected with *Plasmodium falciparum* in the laboratory, but has never been implicated in transmission in nature, although it readily feeds on humans outdoors (De Meillon *et al.*, 1977). This species has been recorded only from South Africa. The rest of the *An. funestus* group are believed to be unimportant in the transmission of malaria parasites due mainly to their zoophilic feeding preferences, but because of the difficulty in identifying them morphologically, often confuse the picture (Gillies & De Meillon, 1968), much as did the *Anopheles gambiae* Giles complex before its resolution in the 1960s (Paterson, 1963; Coluzzi, 1984).

Morphologically, none of the members of the *An. funestus* group can be identified individually with absolute certainty on adult characteristics. Using immature characters, *An. rivulorum* and *An. confusus* Evans & Leeson can be identified on larval characteristics, whereas *An. lesoni* Evans can be identified on both egg and larval morphology (Gillies & De Meillon, 1968). Examination of these characters entails holding wild-caught blood-fed females for egg-laying and rearing of larvae to fourth instar. The latter is by no means simple, as larvae are difficult to maintain under standard insectary conditions. *Anopheles vaneedeni* and *An. parensis* Gillies are both identical to *An. funestus* in almost all respects. Where differences do occur, they are variable and necessitate rearing of family broods to achieve a reasonable probability of accuracy. The other members of the *An. funestus* group either do not occur in southern Africa or are extremely rare (Gillies & De Meillon, 1968).

Cytogenetic studies of the group have shown that *An. funestus*, *An. rivulorum*, *An. lesoni*, *An. parensis* and *An. confusus* each possess unique chromosome inversion rearrangements that can be used to identify them (Green, 1982). *Anopheles vaneedeni*, however, is homosequential, with *An. funestus* differing from it

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only in the possession of a polymorphic inversion on arm 2 (Green & Hunt, 1980). The disadvantages of using cytogenetics as a routine tool for identification purposes are well known. They include sample sizes being limited to half-gravid females which is the only stage of the gonotrophic cycle that contains the giant polytene chromosomes. Unfed females need a blood-meal to initiate the gonotrophic cycle and fully gravid females need to lay eggs before being refed. It is not possible to identify males or immature stages of the *An. funestus* group using cytogenetics. These were among the reasons for developing and testing DNA methods for identifying the *Anopheles gambiae* complex (Paskewitz & Collins, 1990; Scott *et al.*, 1993; Paskewitz *et al.*, 1993; Van Rensburg *et al.*, 1996).

De Meillon *et al.* (1977) realized that the identification of the *An. funestus* group of species would remain a difficult matter until more modern taxonomic techniques could be used. The transmission of malaria in some parts of South Africa, apparently in the absence of the recognized vector *An. arabiensis* Patton, has made finding an alternative identification system a priority. In South Africa *An. funestus* was successfully eradicated in the 1950s through an intensive house-spraying campaign to combat malaria (Hooey, 1974). However, it still occurs in Mozambique, where no control programme has been implemented for many years, and may constitute a threat to those people living in the border regions. During the 1996/97 malaria season in South Africa there was a dramatic increase in densities of the *An. funestus* group in the Komati-poort region bordering on Mozambique, and specimens were collected that were positive for *Plasmodium falciparum* circumsporozoite protein (unpublished data). Unfortunately it was not possible to identify the species because the wild female mosquitoes were immediately processed for ELISA tests

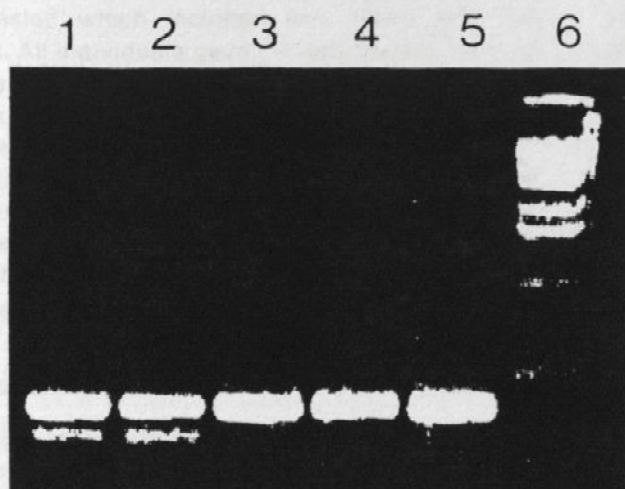
and not allowed to first lay eggs for morphological study as detailed above. This made it imperative that a simple method of identification be developed to establish which member of the *An. funestus* group was responsible for the transmission of malaria in this part of South Africa.

The aim of the present study was to find a method for distinguishing the species of the *An. funestus* group which would be easy to interpret using established laboratory procedures such as those developed for the *An. gambiae* complex.

Results

Family broods of *An. funestus* and *An. vaneedeni* were identified on adult characters as outlined in Gillies & Coetzee (1987). In particular, *An. vaneedeni* adults used in this study were identified by the pale bands at the joints of the tarsomeres which never occur in *An. funestus* (De Meillon *et al.*, 1977; Gillies & Coetzee, 1987).

The molecular procedures were standardized using two *An. funestus* from one Tanzanian and one Madagascan family and one *An. vaneedeni* from a South African family. The D3 amplified product was approximately 390 bp long and the same size fragment was amplified in both species (Fig. 1). PCR yielded products of 100 ng/ μ l or more in the majority of the reactions. Sequencing data revealed a 3% variation between the two species that included four deletions and six substitution mutations. Restriction endonuclease analysis on the *An. funestus* sequence showed 213 recognition sites by 118 different enzymes and the *An. vaneedeni* sequence showed 232 recognition sites by 127 different enzymes. HpaII was chosen on the following criteria: the restriction enzyme must cut the two PCR products at different sites in the two species; these differences



344 - 396 bp

Figure 1. Extracted DNA amplified using D3 primers. Lanes 1-3: *Anopheles funestus* from Tanzania; lane 4: *An. funestus* from Madagascar; lane 5: *An. vaneedeni* from South Africa; lane 6: 1 kb DNA size marker.

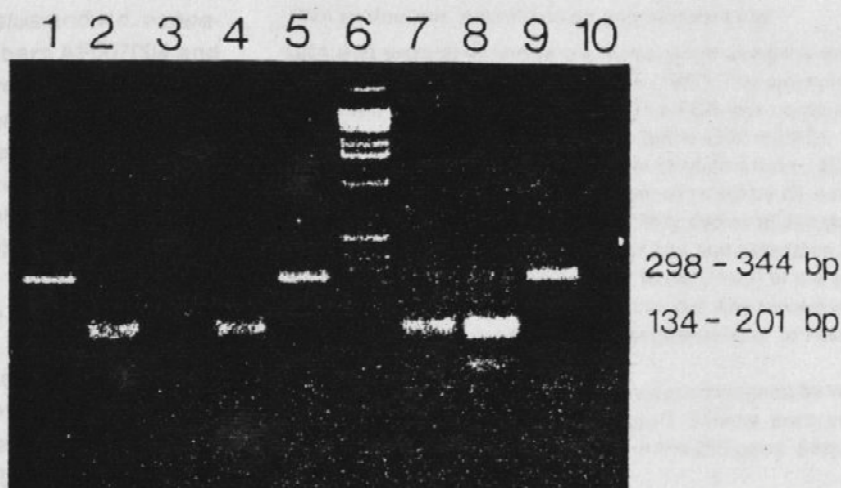


Figure 2. D3 amplified products following digestion with restriction endonuclease HpaII for 1 h at 37 °C using single legs as template DNA. Lanes 1 and 9: *Anopheles funestus* from Tanzania; lane 5: *An. funestus* from Madagascar; lane 10: *An. funestus* from Botswana; lanes 2, 4, 7 and 8: *An. vaneedeni* from South Africa; lane 6: 1 kb DNA size marker; lane 3: negative control.

must be seen without difficulty and without any possible misidentifications. HpaII recognized one site in *An. funestus* approximately 70 bp from the 5' end of the amplified product and *An. funestus* was identified by a 335 bp fragment. *Anopheles vaneedeni* had two HpaII recognition sites, the first approximately 70 bp from the 5' and the second 145 bp downstream from the first site. The first recognition site was identical to the site in *An. funestus*, but *An. vaneedeni* could be distinguished from *An. funestus* by 145 and 170 bp fragments which co-migrated together on a 2% agarose gel and appear as a single band (Fig. 2). The remaining small fragments at 70 bp were not visualized on the gel due either to the small size or the migration of the fragment with the primer dimers.

This technique was tested on eighteen individuals of *An. funestus*, twelve from Madagascar and six from Tanzania. Thirty-two individuals from thirteen egg batches of *An. vaneedeni* from Komatipoort were tested, and in six cases four individuals per batch were tested which included two males and two females. All individuals gave the appropriate banding patterns. When legs from single specimens were used in the amplification process, the same banding patterns were observed after digestion (Fig. 2).

The five specimens from Botswana, which were unidentified either morphologically or chromosomally and labelled only as '*An. funestus* group', were identified with the present technique as being three *An. vaneedeni* and two *An. funestus*.

Discussion

The amplified region between *An. funestus* and *An. vaneedeni* is of approximately the same size and no detectable difference could be seen on a 2% agarose

gel. After HpaII digestion, the RFLP patterns of *An. funestus* and *An. vaneedeni* showed the expected differences.

Incomplete digestion from the PCR product of *An. vaneedeni* gave two bands, the 335 bp band corresponding to the diagnostic band for *An. funestus* and the 145/170 bp band diagnostic for *An. vaneedeni* (Fig. 2, lanes 7 and 8). This could result in misidentification if the concentration of the smaller 145/170 bp band is too low to be visualized on an agarose gel. When 2 h were allowed for restriction to be completed, the diagnostic band was clearly visible. Both DNA extractions and single legs were used in amplification, and although the yield was slightly less when using legs it was still sufficient for use in restriction enzyme digestion.

No intraspecific variation was seen in the samples used here. The amplified region and the restriction endonuclease recognition sites did not reveal any differences between males and females. The mosquitoes tested from Botswana revealed the presence of both *An. vaneedeni* and *An. funestus*, which is a new distribution record for *An. vaneedeni*, having previously been recorded only from South Africa. These specimens were also caught indoors, indicating that *An. vaneedeni* may not be an obligatory outdoor resting species as suggested by the South African data. These identifications do, however, need to be verified, since it is possible that another member of the *An. funestus* group was producing the same fragment of DNA as *An. vaneedeni*. The assay is currently being tested by other researchers on populations of both species from other areas.

Since six species of the *An. funestus* group occur in southern Africa, the development of species-specific diagnostic primers for a PCR protocol has not been attempted at this stage. The small amount of variation

between the sequences of *An. funestus* and *An. vaneeedeni* (see GenBank accession numbers AF007094 and AF007095) may render any such primers obsolete once other species are added to the system. In the meantime the restriction enzyme digestion method appears to be a reliable way of distinguishing between *An. funestus* and *An. vaneeedeni*, although it should be borne in mind that other species of the *An. funestus* group may produce results similar to either of these two species. The method eliminates the problem of having to obtain egg batches from wild females and rear larvae through to adult stage or hold females until half gravid so that polytene chromosome banding sequences can be analysed. In the latter case, since the only chromosomal differences between these two species are polymorphic, a proportion of the specimens will inevitably be unidentifiable. Although this method is more expensive than the others in that sophisticated laboratory equipment and chemicals are needed, it has the advantage that any life stage of the mosquito can be used and field collections are easily stored dry or in alcohol. In addition, now that PCR has become the widely used method of identification for the *An. gambiae* complex (Scott *et al.*, 1993), many laboratories in Africa are equipped to carry out these procedures. Since only one leg of a mosquito is needed for the analysis, the rest of the mosquito can be used for other purposes.

Experimental procedures

Field collections and a priori identifications

Samples used in this study came from the following localities: Madagascar, Ankazobe (18°19'S, 47°07'E), caught biting humans indoors April 1996; Tanzania, Bagamoyo (6°26'S, 38°55'E) at Matimbwa Village resting indoors December 1992; Botswana, Molembo (18°15'S, 21°45'E), Kauxwi (18°17'S, 21°52'E), resting indoors March 1994; South Africa, Komati-poort (25°26'S, 31°57'E), biting humans outdoors January 1996, 1997.

Females were transported to the SAIMR laboratories and held in individual tubes for egg laying. Egg morphology was examined before eggs were put into bowls with aerated water. Larvae were fed on a high protein diet consisting of ground brewer's yeast tablets and dog biscuits. Fourth-instar larvae and pupal pelts were stored initially in ethanol then permanently mounted on microscope slides to be used with pinned adults for morphological identification. The remainder of the adults emerging from individual egg batches were frozen in liquid nitrogen. All specimens were suitably coded so that identification methods could be correlated for individual mosquitoes. Morphological identification was carried out on all reared families according to Gillies & De Meillon (1968) and Gillies & Coetzee (1987). Chromosomal identifications were carried out on Tanzanian *An. funestus* (Green & Hunt, 1980). The samples from Botswana were not identified beyond the morphological grouping of *An. funestus sensu lato*.

DNA extraction, amplification and sequencing

DNA was extracted from single mosquitoes using the standard procedure described in Collins *et al.* (1987). The extracted DNA was used in a 25 µl PCR reaction. The PCR mix contained the following: 2.5 µl of the 10 × reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 1 µM of each primer, 200 µM of each dNTP, 2 U taq DNA polymerase, overlaid by 25 µl mineral oil. Amplification conditions were: thirty cycles of denaturation at 94 °C for 30 s, annealing at 49 °C for 30 s and extension at 72 °C for 30 s, final extension at 72 °C for 10 min. 10 µl of the product was electrophoresed on a 2% agarose gel. The remaining PCR product was later used for either sequencing or in restriction enzyme digestion.

The primers used in amplification were designed by W. Kelly Thomas for use in nematode studies (R. Sharpe, pers. comm.). They amplify a variable D3 domain in the 28S gene. Sequences of the primers are as follows:

D3A sense: 5' GAC CCG TCT TGA AAC ACG GA 3
D3B antisense: 5' TCG GAA GGA ACC AGC TAC TA 3

Approximately 100 ng/µl DNA was used in the sequencing reaction. PCR products were pretreated using exonuclease I and shrimp alkaline phosphatase before the sequencing reaction was performed (Amersham Life Science, cat. no. US70170). The sequence of the D3 amplified product was aligned with other sequences in GenBank to confirm that the correct product was amplified. Sequencing data from one *An. vaneeedeni* and two *An. funestus* individuals were analysed using the laser gene program and submitted to GenBank with accession numbers of AF 007095 and AF 007094 respectively. HpaII restriction endonuclease was chosen to discriminate between these two species. 10–15 µl PCR products were restricted for 2 h at 37 °C with HpaII (Boehringer Mannheim, cat. no. 239291) after confirmation of amplification by electrophoresis on a 2% agarose gel.

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Chapter 5

SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP)

5.1 INTRODUCTION

Single-strand conformation polymorphism (SSCP) is based on the principle that single-strand DNA (ssDNA) has the ability to form secondary structure (conformations) depending on the nucleotide sequence of that strand (Dieffenbach and Dveksler, 1995). Differences in sequences will result in the formation of different conformations or secondary structures. These different conformations can be separated by electrophoresis. At this stage it is not possible to predict what the effect of a mutation will have on the mobility during electrophoresis (Hayashi, 1991). SSCP electrophoresis appears to be more sensitive in detecting mutations in smaller fragments (20-400bp) than longer fragments (Hayashi, 1991). Longer fragments (>400bp) can be restricted using restriction endonuclease and the smaller restricted fragments then electrophoresed.

This method is very sensitive and differences as small as a single base difference at the sequence level can show a mobility shift when SSCP analysis is performed (Orita *et al.*, 1989a). Approximately 97% of mutations can be detected by mobility shifts in glycerol gels providing that the DNA fragment is between 100-300bp, while 67% of mutations can be detected in 300 - 450 bp DNA fragments (Hayashi, 1991). Hayashi translated these values back to the sensitivity of the PCR-SSCP assay, using a mathematical calculation, which resulted in a 99% and 89% sensitivity for 100-300bp and 300-450bp respectively.

A particular ssDNA can show at least two different molecular shapes, depending on the conditions of the electrophoresis (Orita *et al.*, 1989b). These molecular shapes are visualized as bands on a stained SSCP gel. One band represents the denatured ssDNA and the other band the renatured ssDNA. For a denatured dsDNA molecule at least four different molecular shapes are possible, which are represented as four bands on a SSCP gel. However when the sequence difference results in the same conformation, the polymorphism will not be detected by SSCP analysis (Orita *et al.*, 1990).

The conformation of ssDNA can be altered by changing the physical parameters such as temperature, ionic strength or by the addition of a denaturant (glycerol) within the gel matrix. The ratio between the concentration percentage of N,N'-methylenebisacrylamide and acrylamide (%C, which is the ratio of the percent concentration on N,N'-methylenebisacrylamide to the concentration of total acrylamide monomer) determines the degree of cross-linking within a polyacrylamide gel matrix. Complementary ssDNA are separated with more sensitivity in gels with low cross linking. A 1- 2 %C is generally used with a 5-6% total acrylamide concentration. The addition of 5-10% glycerol to the gel solution opens up the renatured ssDNA molecule and appears to assist in detecting polymorphism, but is not a rule in all mutation studies (Hayashi, 1991).

SSCP analysis involves the denaturing of a amplified DNA fragment followed by immediate cooling of denatured ssDNA fragments to assist in the renaturing of ssDNA to form secondary structures, rather than the reannealing of the complementary strand of DNA. Electrophoresis through a neutral polyacrylamide gel separates the different molecules from each other on the basis of size and conformation. These different conformations can be visualised using either radioactivity, ethidium bromide or silver staining (Hayashi, 1991; Yap and McGee, 1992).

Silver staining and ethidium bromide are less hazardous to use than radioactivity and are as sensitive in detecting polymorphisms. An advantage of a PCR-SSCP assay is that the samples need minimal handling before electrophoretic separation. The primers used in amplification of a particular DNA fragment generally flank a variable region and the primer sequence itself is located within conserved regions.

Since 1989 when Orita *et al.* (1989a) first described the use of SSCP analysis, this method has become widely used for the detection of DNA mutations. SSCP polymorphism has been used for the construction of high resolution linkage maps, the identification of genotypes of individuals for clinical and other purposes (Orita *et al.*, 1990). SSCPs can also be used as a diagnostic tool to detect drug resistance in *Mycobacterium tuberculosis* and is far more rapid than conventional resistance testing (Telenti *et al.*, 1993). Hiss *et al.* (1994) used SSCP analysis to separate five species of *Aedes* mosquitoes belonging to the subgenus *Ochlerotatus*. SSCP was used to differentiate three genera of the Triatominae (Heteroptera: Reduviidae) and were used in detecting mutations causing human diseases such as neurofibromatosis, cystic fibrosis and Tay-sachs disease (Cawthon *et al.*, 1990; Dean *et al.*, 1990; Ainsworth *et al.*, 1991; Fukai *et al.*, 1995).

Areas selected for sequencing in *An. funestus* could not successfully be used to develop a PCR assay and SSCP analysis was examined as an alternative method to identify members of the group. The same primer pair was used to sequence the D3 variable domain on the 28S gene described in Chapter 3, was used for the SSCP analysis.

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5.2 MATERIALS AND METHODS

PCR amplification

PCR conditions were standardised to obtain amplicons for all four species. The D3 primer pair was used to amplify the variable domain within the 28S gene on the rDNA. The final PCR condition was as follows: 10X reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 10X dNTPs (200 μ M of each dNTP), 1.5mM MgCl₂, 1 μ M of each primer, 2 units of *Taq* polymerase. PCR cycles were as follow: denaturing at 94°C for 30 seconds, annealing at 40°C for 30 seconds, extension at 72°C for 30 seconds and the final autoextension for 72°C for 5min. The amplicon had an approximate size of 390bp and all species gave the same size product after amplification. The amplified products (5 μ l-6 μ l) were loaded on 2-2.5% agarose gel and electrophoresed for 1hour to determine the success of amplification. Those with low yield, or none at all, were excluded in the SSCP analysis and were reamplified during the next round of tests. Negative controls included all reagents used in the PCR (cocktail) mixture (excluding the DNA template) plus a negative DNA extraction control which was added to the PCR mix in the same quantities as the DNA. DNA templates were obtained from DNA extractions (see Chapter 3 under Materials and Methods) or from single legs. Amplified samples ready for SSCP analysis were stored in either the fridge or freezer until they were electrophoresed.

SSCP electrophoresis

Different electrophoretic systems were tried in order to obtain the best separation between the different conformations. Two different acrylamide solutions were compared, 5%-6% acrylamide solution (49:1; acrylamide :bisacrylamide) and a Mutation Detection Enhancement (MDE™) gel solution supplied by FMC Bioproducts. Two different electrophoresis apparatus were compared, firstly the sequencing 20 x 40cm apparatus with 0.4mm gels, and secondly the Hoeffer SE600

series (16 x 18cm) with 1mm thick gels. Different temperatures were tested when using the Hoeffer SE600 series, because of the presence of a central cooling core. Temperatures settings were 0°C, 10°C, 15°C and 20° C. The temperature of the gel was approximately 4°C higher than the setting on the cooling apparatus. Electrophoresis was done overnight using 0.6X TBE electrode buffer for 16-18 hours if the MDE™ solution was used at a constant voltage of 200V. The time for electrophoresis was much shorter (3-6 hours) if polyacrylamide solution (49:1) was used. After electrophoresis the gel was removed and stained immediately either by using a silver staining procedure or by using ethidium bromide.

Table 5.1 Specimens used in SSCP analysis of four members of the *Anopheles funestus* group.

Country	Locality	No of families	No of individuals	Species
Tanzania	Matimbwa	3	16	<i>An. funestus</i>
	Bagamoya	1	1	<i>An. funestus</i>
Côte d'Ivoire	Petionara	3	8	<i>An. funestus</i>
	Abidjan	2	6	<i>An. funestus</i>
Senegal	Kedougou	-	6	<i>An. funestus</i>
	Sankagne	-	6	<i>An. funestus</i>
	Dielmo	-	6	<i>An. funestus</i>
Mozambique	Boane	8	26	<i>An. funestus</i>
Madagascar	Ankazobe	9	27	<i>An. funestus</i>
Botswana	Shakawe	1	6	<i>An. funestus</i>
South Africa	Mamfene	1	1	<i>An. rivulorum</i>
	Mamfene	10	10	<i>An. vaneedeni</i>
	Tzaneen	2	2	<i>An. lesoni</i>
	Tzaneen	16	38	<i>An. vaneedeni</i>
	Komatipoort	7	30	<i>An. vaneedeni</i>
	Komatipoort	3	20	<i>An. rivulorum</i>

Staining

Silver staining as described in Budowle *et al.* (1991) was used. The gel was soaked in a 10% ethanol solution. It was found that analar grade absolute ethanol was necessary in order to avoid producing a dark background that could obscure the SSCP bands. The gel was then oxidized in 1% nitric acid followed by staining in 0.012M AgNO₃ and was reduced using 0.28M sodium carbonate with 750 μ l formaldehyde solution. Budowle *et al.* (1991) used 0.019% formalin in the original staining procedure. The reaction was stopped by adding 10% acetic acid as soon as the desired band intensity was observed. Before the gel was dried it was soaked in 50% ethanol and 5% glycerol solution for 30 min to prevent cracking after drying. Filter paper was placed over the gel in order to lift it from the glass plate or from the plastic container. The gel was dried overnight and then transferred onto a transparency by the use of clear adhesive plastic film. This storage method was found to be effective and economical.

Ethidium bromide stain was also performed by soaking the gel for 20 min in 1X TBE buffer containing 3 μ l (10mg/ml) ethidium bromide/ 100ml buffer. The gel was exposed under an ultra violet light and photographed for permanent record.

5.3 RESULTS

The results of this study have been published and a copy of the manuscript has been attached at the end of this chapter (see Acknowledgements at the beginning of this thesis for the contributions of each author). What follows is a description of the procedures followed in order to reach the results that were eventually published.

Four species within the *An. funestus* group were analysed using SSCP electrophoresis to find species-specific banding patterns. Amplification methods were adapted from those mentioned in Chapter 3 in order to amplify all species within the *An. funestus* group using the same PCR conditions. Amplification produced DNA fragments of nearly 400bp long.

Comparisons between the two different acrylamide solutions showed that the MDE™ solution gave better band resolution than the polyacrylamide solution (49:1). Electrophoretic temperature at 15°C gave the best resolution between conformation forms. No difference in polymorphisms were observed when comparing the Hoeffer SE600 series and the sequencing gel apparatus (40cm x 20cm) with both giving satisfactory results.

Double strand DNA does not appear on MDE™ gels electrophoresed overnight, because dsDNA migrates so fast that the molecules migrate out of gel matrix. The use of polyacrylamide solution resulted in only one band visible for the RSS and DSS molecules, suggesting that the two RSS or two DSS molecules are migrating together and the resolution of gel matrix is not sufficient to separate them into two distinct bands.

The size-marker (1Kb) loaded on each gel was only used as an indication of the order of sample loading with the size marker as sample number one. Each gel contained amplified samples from morphological or chromosomal identified species and the unknown specimens were compared with the standards to get identification. Silver staining is more time consuming than ethidium bromide, but has the advantage of having no toxic waste that needs disposal, and expensive photographic equipment is not needed for a permanent record of the gels.

5.4 DISCUSSION

The mutation detection electrophoresis gel solution from FMC Bioproducts gave a much better resolution than the 49:1 acrylamide solution tested. Additional polymorphisms were not observed when using the MDE™ solution in comparison to the acrylamide solution, but it was not possible to visualize the individual DSS or RSS molecules when the acrylamide solution was used. Temperatures of 15°C gave better resolution than at 4°C when limited resolution was observed.

The additional F-R3 band (see Figure 2 in the publication) in *An. funestus* specimens from Kedougou and Sankagne, Senegal can be explained as sequence polymorphism occurring within these specimens. The presence of the polymorphism can be used as a marker to distinguish between different populations where this polymorphism occurs. Only specimens from Dielmo in Senegal showed typical East African *An. funestus* banding patterns without the additional F-R3 band being present. Lochouart *et al.* (1998) published results showing that at least two distinct *An. funestus* populations are present in Senegal. The one population is highly anthropophilic with a high degree of malaria parasite infection rate and the other population is less anthropophilic with a lower infection rate. *Anopheles funestus* from Dielmo village were highly anthropophilic and had the standard chromosomal arrangement (Green and Hunt, 1980). Mosquitoes tested for bloodmeal analysis were caught in bedrooms and by comparison with other villages human hosts were as freely available as horses. Ninety one percent of *An. funestus* from Dielmo fed on human hosts.

The population from Kedougou had chromosomal inversion arrangements different to that seen

in the Dielmo population. Blood meal analysis from Kedougou specimens showed that 81% of specimens fed on humans while 21% fed on bovine blood. *Anopheles funestus* from Sankagne showed 25% of specimens fed on horses and 74% fed on human hosts. Sankagne samples showed yet another configuration of the chromosomal banding arrangements. Lochouart *et al.* (1998) speculate that in fact two gene pools are present, one in Sankagne and the other at Wassadou and Kedougou and that these populations show reproductive isolation when in sympatry. My results, however, indicate the Dielmo population should also be considered distinct from the other two, meaning that *An. funestus* in Senegal could comprise a complex of three biological species.

It is important that standards of all the species be used in each SSCP gel, because variation might occur between different electrophoretic reactions and additional polymorphisms that might occur can be related to the standards. The Department of Medical Entomology, SAIMR., will act as a repository for DNA samples of each species that can be provided to researchers who want to use the assay. It is recommended that wild specimens used for SSCP analysis be identified using other methods such as polytene chromosomes or morphology, since the SSCP assay has not yet been validated on large sample sizes or from regions where no studies have been carried out on the *An. funestus* group.

Single-Strand Conformation Polymorphism Analysis for Identification of Four Members of the *Anopheles funestus* (Diptera: Culicidae) Group

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ABSTRACT Members of the *Anopheles funestus* Giles group are difficult to identify because of the morphological overlap that exists within the group. This inability to distinguish species, as well as the fact that the species vary in their behavior and biting preferences, complicate the successful planning and maintaining of malaria control programs. In this article we discuss the use of a single-strand conformation polymorphism (SSCP) assay to distinguish 4 members of the *An. funestus* group collected at 10 different localities in Africa. rDNA genes differ at numerous sites among closely related species. Using conserved primers, the D3 domain in the 28S gene was amplified, electrophoresed on SSCP gels, and species-specific patterns were observed. Intraspecific variation was detected in *An. funestus* specimens from East and West Africa. Analyzing 108 *An. funestus*, 78 *An. cameroni* Gillies & Coetzee, 21 *An. rivulorum* Leeson, and 2 *An. lesoni* Evans, we concluded that SSCPs can be used successfully as a molecular tool for the identification of these species.

KEY WORDS: *Anopheles funestus* group, identification, single-strand conformation polymorphism analysis

SPECIES COMPLEXES HAVE been shown to exist in several groups of medically important Diptera. Complexes are groups of species that are similar morphologically but exhibit behavioral and vectorial differences. One problem in vector control today is the difficulty of identifying the cryptic species within these groups or complexes. This identification is necessary because not all the species within these groups are involved equally in the transmission of pathogens to humans. A classical example is the *Anopheles gambiae* Giles complex, where only 2 of a total of 7 recognized species are major vectors of the malaria parasite *Plasmodium falciparum* (Gillies and deMeillon 1968, Service 1985, Gillies and Coetzee 1987, Hunt et al. 1998). Rapid large-scale identification of the species in this complex was greatly improved by the development of the species-specific polymerase chain reaction (PCR) assay (Scott et al. 1993). This technique is routinely used in many laboratories to identify members of the *An. gambiae* complex (Paskewitz et al. 1993, Van Rensburg et al. 1997, Temu et al. 1998, Toure et al. 1998). The 3rd major vector species of malaria in sub-Saharan Africa belongs to another group of species, the *Anopheles funestus* Giles group.

The *An. funestus* group consists of at least 8 species—the nominal vector *An. funestus*, *An. cameroni* Gillies & Coetzee, *An. rivulorum* Leeson, *An. lesoni* Evans, *An. confusus* Evans & Leeson, *An. parensis* Gillies, *An. brucei* Service, and *An. arabi* Sobti. *An. funestus* has been recognized as a major vector of malaria parasites since the early part of the 20th century (Gillies and De Meillon 1968). The only other member of the group that has been found to be infected naturally with *P. falciparum* is *An. rivulorum* (Wilkes et al. 1996). This species is widespread in Africa and is usually zoophilic. However, if cattle are scarce or not available, this species will feed on humans and can be infected naturally with *P. falciparum*. *Anopheles cameroni*, occurring in southern Africa, has been infected with *P. falciparum* in the laboratory but has not been found naturally infected with the parasite (De Meillon et al. 1977, Gillies and Coetzee 1987).

The major problem when working with the *An. funestus* group is the difficulty in identifying the different species. Morphological characteristics of the adults are variable and overlap in many instances. *An. cameroni* and *An. parensis* can be separated from *An. funestus* by, if present, pale scaling on the tarsomeres and male palps respectively (Gillies and Coetzee 1987). Larval morphology can be used to identify *An. confusus* and *An. rivulorum*, and *An. lesoni* can be identified on the basis of both larval and egg morphology (Gillies and De Meillon 1968, Gillies and Coetzee 1987). Polytene chromosome banding arrangements can distinguish *An. funestus*, *An. rivulorum*, *An. lesoni*, *An. parensis*, and *An. confusus* from one another

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(Green 1982). However, *An. funestus* and *An. vaneevii* have homosequential banding patterns and differ by a single floating inversion on arm 2 (Green and Hunt 1980). Polytene chromosomes are found in the ovaries of half-gravid females or the salivary glands of the 4th instars, and the technique needs a great deal of expertise to analyze the results. Morphological identification however, needs all the life stages of the mosquito (from the eggs to the adult). This method is time-consuming and mortality is high when rearing isofemale lines under laboratory conditions.

Molecular techniques have the advantage of being rapid and inexpensive, applicable to all life stages and, for a PCR assay, limited material is needed. To date only one molecular DNA-based method exists to identify members of the *An. funestus* group, that being a restriction fragment-length polymorphism (RFLP) assay for distinguishing *An. funestus* from *An. vaneevii* (Koekemoer et al. 1998).

Single-strand conformation polymorphism (SSCP) is a technique that has been used recently as a molecular taxonomic tool. Hayashi (1991) gave a good overview of the practical use of SSCP electrophoresis and found that mutated clones with the same SSCP banding pattern have the same sequence mutations. Hiss et al. (1994) used SSCP to show that species-diagnostic banding patterns could be achieved for various taxa by this relatively new technique.

The length, location, and number of intrastrand base pairs determine the secondary and tertiary structure of a conformation of single-stranded DNA (Orita et al. 1989). SSCP electrophoresis is based on this principal, and the difference in the DNA sequence will alter the conformation of single-strand DNA that will result in different mobilities during electrophoresis. The PCR products are denatured and then directly plunged into ice to help in the formation of intrastrand complexes and reducing the renaturing of complementary strands. Formamide dye and a neutral polyacrylamide gel help to maintain the molecules secondary conformation during electrophoresis (Hiss et al. 1994).

Currently it is not possible to predict the mobility shift because of mutation differences, however shorter fragments (100–400 bp) are more sensitive for the detection of mutation variation (Hayashi 1991). Mutations incorporated following speciation should be readily detected according to Hiss et al. (1994). Electrophoresis conditions (e.g., temperature changes and the concentration of ions and solvents) may influence the sensitivity of mutation detection, and the electrophoresis process must be standardized for each set of conditions. SSCP electrophoresis has been used in different mutation studies such as in the human genetic field for detection of human genetic diseases and genetic markers (Orita 1989) and in phylogenetic studies of wheat and grass (Wang 1997).

Here we describe the use of SSCPs for distinguishing between 4 members of the *An. funestus* group.

Materials and Methods

Field Collections and a priori Identifications. The samples used in this study came from the following 7 countries: (1) Madagascar, Ankazobe (18° 19' S, 47° 07' E), caught biting humans indoors April 1996; (2) Tanzania, Bagamoyo (6° 26' S, 38° 53' E) at Matimbwa Village resting indoors December 1992; (3) Botswana, Shakawe (18° 10' S, 21° 43' E), resting indoors March 1994; (4) South Africa, Komatipoort (25° 26' S, 31° 57' E), biting humans outdoors and CO₂-baited net traps, January 1996, 1997; Tzaneen, Thomo Camp, Giyani (23° 15' S, 30° 47' E), pit trap December 1997, Mamfene (27° 23' S, 32° 16' E), feeding on cattle March 1998; (5) Mozambique, Boane (26° 02' S, 32° 19' E), caught resting indoors, May 1997; (6) Côte d'Ivoire, Pétionara (near Bouake) (7° 42' N, 5° 0' W) caught inside houses, June 1997; Abidjan (5° 19' N, 4° 1' W) caught inside houses, June 1997; (7) Senegal, caught indoors using pyrethrum spray catch: Dielmo (13° 45' N, 16° 25' W), February 1998; Kedougou (12° 33' N, 12° 11' W) October 1997 and Sankagne (13° 24' N, 13° 45' W), November 1997.

All samples, except those from Senegal, were transported to the laboratory where females were held in individual tubes for egg laying. Egg morphology was examined before eggs were put into bowls with aerated water. Larvae were fed on a high-protein diet consisting of ground brewers' yeast stored and dog biscuits. Larval and pupal pelts were stored initially in 80% ethanol, then permanently mounted on microscope slides to be used with pinned adults for morphological identification. The remainder of the adults emerging from individual egg batches were frozen in liquid nitrogen. Morphological identification was carried out according to Gillies and De Meillon (1968) and Gillies and Coetzee (1987). The Senegal samples were identified chromosomally (Green and Hunt 1980).

DNA Extraction, Amplification, and Sequencing. The primers used in amplification were designed by W. Kelly Thomas for use in nematode studies (R. Sharpe, Leeds University, UK, personal communication). They amplify a variable D3 domain in the 28S gene. Sequences of the primers are as follows:

D3A sense: 5' GAC CCG TCT TGA AAC ACC GA 3'

D3B antisense: 5' TCG GAA CGA ACC ACC TAC TA 3'

Amplifications were done using the template from DNA extractions (Collins et al. 1987) or from single legs centrifuged at 16,000 × g for 4 min with the PCR mixture. The PCR mixture contained the following: 2.5 µl of the 10x reaction buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3), 1.5 mM MgCl₂, 1 µM of each primer, 200 µM of each dNTP, 2 U thermostable taq DNA polymerase, overlaid by 30 µl of mineral oil. Amplification conditions were 30 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s, and extension at 72°C for 30 s, final extension at 72°C for 10 min. 5 µl of the product was electrophoresed on a 2% agarose gel. The remaining PCR product was later used for SSCP analyses.

Two negative controls were included. The 1st was a DNA extraction negative control where extractions were performed without any DNA. The 2nd control was a negative control containing all the substances in the PCR mixture except DNA template. The positive control for the SSCP electrophoresis was obtained by amplifying extracted DNA from 1 *An. finestus* from Tanzania which was chromosomally identified (Green and Hunt 1980). DNA from this specimen was used in every gel throughout the trials.

SSCP Electrophoresis. SSCP electrophoresis was done according to Hiss et al. (1994), with minor alterations. Mutation Detection Enhancement gel solution, 2 times concentration from FMC Bioproducts, Rockland, ME, (catalog number 50620) was diluted to a 0.5-times concentration and used for gel casting. Electrophoresis was performed using a Bio-Rad PROTEIN II xi 2-d Cell (16 by 18 cm) and with 1-mm-thick gels. Glass plates were prepared according to manufacturer's specifications. Different electrophoresis temperatures were tested at 4, 15, and 20°C. The central cooling core helped in keeping temperatures at the correct settings. Before denaturing, 3 µl formamide was added to 10 µl PCR product. The centrifuge tubes (0.5 ml) were put into ice immediately after denaturing and 10 µl of PCR product was electrophoresed for ≈16 h at 200V. The electrode buffer was 0.6 M TBE and a final concentration of TBE in the gel was 0.05 M. A 1Kb-size marker was loaded in the 1st lane on the left-hand side to show the orientation of the gel.

Gels were stained using a general silver staining technique as described in Bidowle et al. (1991) and fixed in 10% acetic acid when the desired intensity was reached. Gels were dried in the air or in a heated vacuum drier. Chemicals used for staining, except sodium carbonate and acetic acid, could be reused for up to 3 times without loss of sensitivity.

Results

The morphologically identified species and the number of specimens used in this assay are given in Table 1. All *An. cameroni* specimens were identified by the pale banding on the tarsomeres, whereas *An. finestus* had only black scaling on the legs (De Meillon et al. 1977). *Anopheles rivulorum* and *An. leesonii* were identified on larval and egg characteristics (Gillies and DeMeillon 1968).

The D3 primers amplified the same fragment of ≈400 bp, and no obvious size differences were seen among the 4 species when visualized on a 2% agarose gel stained with ethidium bromide. The optimum temperature for electrophoretic separation was at 20°C, and at 4°C, resolution seemed to decrease.

Figure 1 illustrates the species-specific banding patterns observed after SSCP electrophoresis. The upper set of bands represent renatured single-strand (RSS) DNA and the lower set of bands represents the denatured single-strand (DSS) DNA (Hiss et al. 1994). Renatured double-strand DNA, that would have appeared between the RSS and DSS molecules, was never visualized on our gels. For each species, at least

Table 1. Specimens used in SSCP analysis of 4 members of the *An. finestus* group.

Country	Locality	No. families	No. individuals	Species
Tanzania	Matumbwa	3	16	<i>An. finestus</i>
	Bagamoyo	1	1	<i>An. finestus</i>
Côte d'Ivoire	Pétionara	3	8	<i>An. finestus</i>
	Abidja	2	6	<i>An. finestus</i>
Senegal	Kedougou	—	6	<i>An. finestus</i>
	Sankagne	—	6	<i>An. finestus</i>
	Dielmo	—	6	<i>An. finestus</i>
Mozambique	Boane	8	26	<i>An. finestus</i>
Madagascar	Ankazobe	9	27	<i>An. finestus</i>
Botswana	Shakawe	1	6	<i>An. finestus</i>
South Africa	Manifene	1	1	<i>An. rivulorum</i>
	Manifene	10	10	<i>An. cameroni</i>
	Tzaneen	2	2	<i>An. leesonii</i>
	Tzaneen	16	38	<i>An. cameroni</i>
	Komatipoort	7	30	<i>An. cameroni</i>
	Komatipoort	3	20	<i>An. rivulorum</i>

4 different bands were observed. These are labeled with the 1st letter of each species name (e.g., F = *finestus*, V = *cameroni*, etc.), an R or a D to designate renatured or denatured single-strand DNA, and the numbers 1, 2, or 3 to indicate mobility. The RSS bands were used as the diagnostic banding pattern to identify each species. The *An. finestus* F-R1 and F-R2 bands were constant and always present in the 108 specimens from 10 different localities. Intraspecific variation was noted between specimens from East and West Africa, where an additional molecule F-R3 always was absent in the East African samples but present in all of the 14 specimens tested from the 2 localities in Côte d'Ivoire and in 12 of the 18 specimens from Senegal (Fig. 2). The F-R3 molecule was slightly slower than the V-R2 molecule and did not influence identification. The F-R1 molecule was always present, and the F-R3 and F-R1 molecules were half the intensity of F-R2. *An. finestus* from Mozambique had an

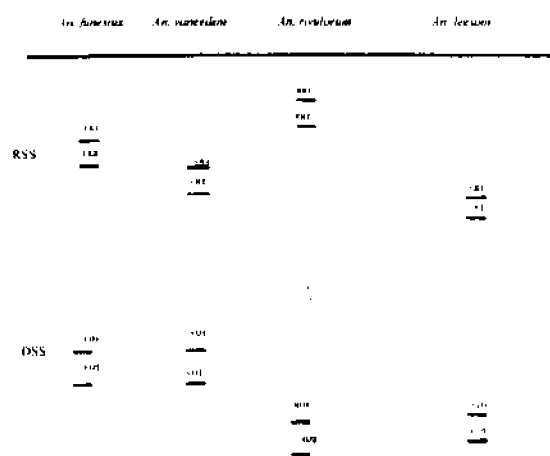


Fig. 1. Species-specific banding patterns observed after SSCP electrophoresis of 4 members of the *An. finestus* group. RSS, renatured single-strand; DSS, denatured single-strand; F, *finestus*; V, *cameroni*; R, *rivulorum*; L, *leesonii*; R1 and R2, RSS molecules; D1 and D2, DSS molecules.

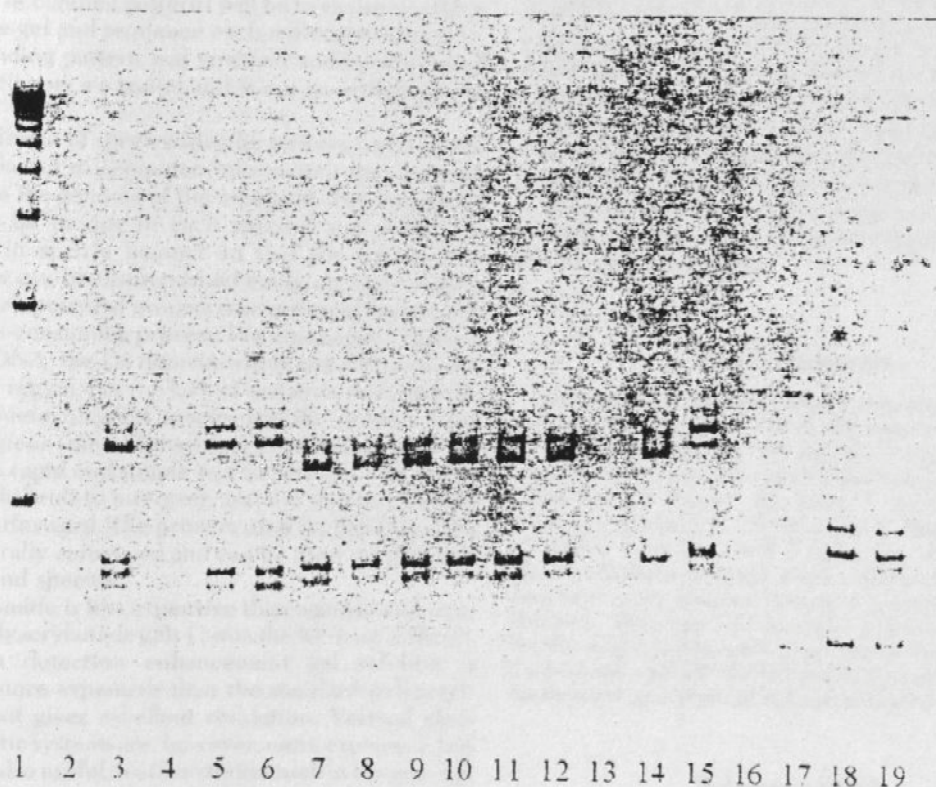


Fig. 2. SSCP analysis showing intraspecific and interspecific variation. Lane 1, 1Kb-size marker; 2, negative control; 3, *An. funestus* (Mozambique); 5, *An. funestus* (Tanzania); 6, *An. funestus* (Madagascar); 7-11, *An. vaneeedeni* (South Africa, Tzaneen); 12,14, *An. vaneeedeni* (South Africa, Komatipoort); 15, *An. funestus* (Côte d'Ivoire, Abidjan); 16-17, *An. rivulorum* (South Africa); 18-19, *An. leesonii* (South Africa). Lanes 4 and 13 did not amplify.

additional F-D molecule (F-D1a) which was half the intensity of the F-D2 molecule and migrated more slowly than F-D1. Other than that, the DSS molecules of *An. funestus* and *An. vaneeedeni* migrated at the same speed. The RSS molecules of *An. funestus* from Mozambique did not show intraspecific variation when compared with other *An. funestus* from different regions.

Twenty-one *An. rivulorum* tested showed the same banding patterns and without any variation in either DSS or RSS molecules. Of the 75 *An. vaneeedeni* tested, none showed any intraspecific variation, and the banding patterns were constant between runs. Only 2 *An. leesonii* specimens were collected for analysis. Amplification and electrophoresis of these 2 were repeated independently on 5 occasions and the same results were obtained each time. One of the 2 individuals displayed intraspecific variation where L-R3 was present with the L-R1 and L-R2 molecules.

The diagnostic bands for each species are depicted diagrammatically in Fig. 1. Figure 2 shows actual specimens of all 4 species. Migration of the size marker molecules was not constant, and variation was observed when the duration of the electrophoresis run was longer than 16 h.

Discussion

For each single-strand molecule at least 2 different bands will be observed, and for a denatured double-strand molecule at least 4 bands will be visible (Orita et al. 1989). Two of the 4 bands will correspond to the complementary strand and their mobilities will be different because of a different structural folding. Species-specific patterns always were observed with the RSS molecules. Because of the overlap between the DSS molecules of *An. funestus* and *An. vaneeedeni*, these sets alone should not be used for species diagnosis. However, both the RSS banding pattern and DSS banding patterns were distinctive for *An. rivulorum* and *An. leesonii*. The RSS banding patterns were stable and no variation between R1 and R2 molecules of the same species were observed except the additional R3 molecule in *An. funestus* specimens from West Africa. This, however, never compromised the usefulness of the SSCPs for identification purposes. There might be sequence differences in West African populations that result in forming an additional conformation. The fact that the F-R1 and F-R3 are both of the same intensity and half the intensity of F-R2 suggest that F-R1 and F-R3 molecules are from the same complementary strand. Their sequence might permit them to form

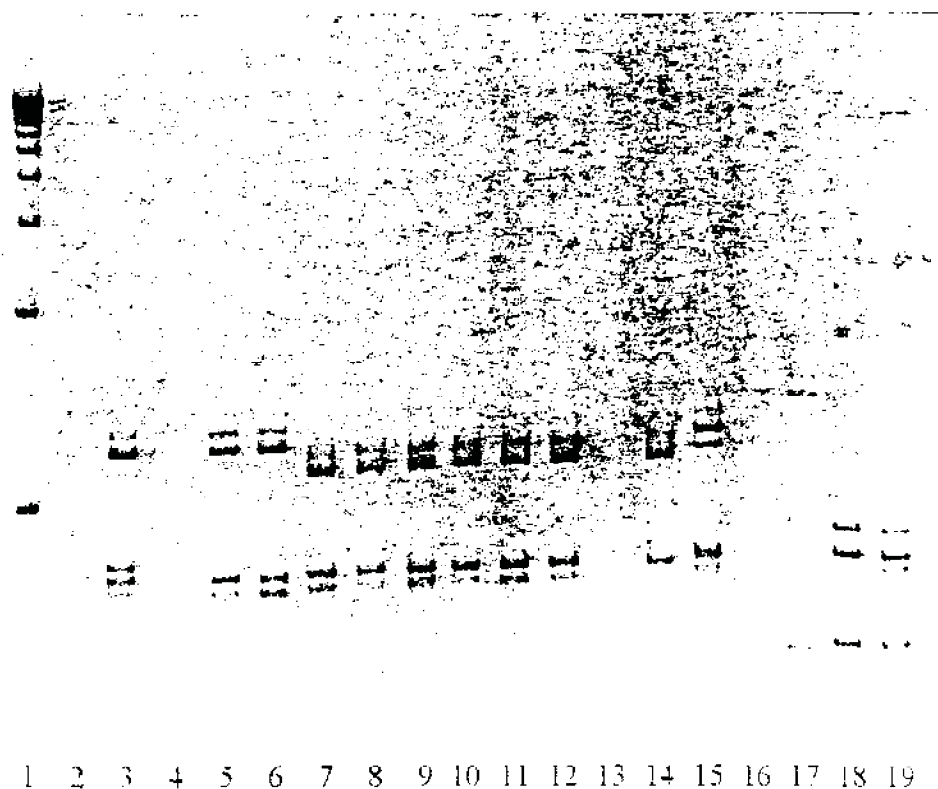


Fig. 2. SSCP analysis showing intraspecific and interspecific variation. Lane 1: 1 Kb-size marker; 2, negative control; 3, *An. funestus* (Mozambique); 5, *An. funestus* (Tanzania); 6, *An. funestus* (Madagascar); 7-11, *An. cameroensis* (South Africa, Tzaneen); 12-14, *An. cameroensis* (South Africa, Komatipoort); 15, *An. funestus* (Côte d'Ivoire, Abidjan); 16-17, *An. crucians* (South Africa); 18-19, *An. leesonii* (South Africa). Lanes 4 and 13 did not amplify.

additional F-D molecule (F-DL₂) which was half the intensity of the F-D₂ molecule and migrated more slowly than F-D₁. Other than that, the DSS molecules of *An. funestus* and *An. cameroensis* migrated at the same speed. The RSS molecules of *An. funestus* from Mozambique did not show intraspecific variation when compared with other *An. funestus* from different regions.

Twenty-one *An. viciniorum* tested showed the same banding patterns and without any variation in either DSS or RSS molecules. Of the 75 *An. cameroensis* tested, none showed any intraspecific variation, and the banding patterns were constant between runs. Only 2 *An. leesonii* specimens were collected for analysis. Amplification and electrophoresis of these 2 were repeated independently on 5 occasions and the same results were obtained each time. One of the 2 individuals displayed intraspecific variation where L-R₃ was present with the L-R₁ and L-R₂ molecules.

The diagnostic bands for each species are depicted diagrammatically in Fig. 1. Figure 2 shows actual specimens of all 4 species. Migration of the size marker molecules was not constant, and variation was observed when the duration of the electrophoresis run was longer than 16 h.

Discussion

For each single-strand molecule at least 2 different bands will be observed, and for a denatured double-strand molecule at least 4 bands will be visible (Ortiz et al. 1999). Two of the 4 bands will correspond to the complementary strand and their mobilities will be different because of a different structural folding. Species-specific patterns always were observed with the RSS molecules. Because of the overlap between the DSS molecules of *An. funestus* and *An. cameroensis*, these sets alone should not be used for species diagnosis. However, both the RSS banding pattern and DSS banding patterns were distinctive for *An. viciniorum* and *An. leesonii*. The RSS banding patterns were stable and no variation between R₁ and R₂ molecules of the same species were observed except the additional R₃ molecule in *An. funestus* specimens from West Africa. This, however, never compromised the usefulness of the SSCP for identification purposes. There might be sequence differences in West African populations that result in forming an additional conformation. The fact that the F-R₁ and F-R₃ are both of the same intensity and half the intensity of F-R₂ suggest that F-R₁ and F-R₃ molecules are from the same complementary strand. Their sequence might permit them to form

either 1 of the 2 conformations and with the same bias. However, the only way to know what causes the difference in banding patterns will be to excise the DNA from the gel and sequence each molecule separately. The banding pattern was constant and present in all the specimens we tested and is not an artifact of the PCR.

The design of species-specific primers involves the accumulating of sequence information on a certain region in the genome of the mosquito. The sequences need to be unique in each species, and should be located in such a manner so that the unique size products can be distinguished easily on agarose gels. The development of primers is an extremely expensive and time-consuming process. We sequenced 2 regions in the rDNA, the D3 domain within the 28S gene and the ITS1 region, but the lack of variation excluded the development of good species-specific primers from these regions (unpublished data). SSCP analysis, however, is a rapid and simple test to perform, the results are not difficult to interpret, and the staining method is straightforward. The primers used for SSCP analysis are generally conserved and can be used for different genera and species.

Acrylamide is less expensive than agarose and casting of polyacrylamide gels (1 mm thick) is not difficult. Mutation detection enhancement gel solution is slightly more expensive than the standard polyacrylamide, but gives excellent resolution. Vertical electrophoretic systems are, however, more expensive, but they are also useful in other studies such as iso-enzyme for population genetic studies. Time needed for electrophoresis is much longer (16 h), but this does not interfere with working hours. SSCP gels can be visualized using radioactivity, fluorescence, or silver staining. Processing radioactive waste is problematic and the danger involved in working with radioactivity puts this method at a disadvantage. Fluorescence must be kept in the dark and the shelf life is short. Silver staining is easy to use and relatively inexpensive. Hiss et al. (1994) showed that silver staining was as sensitive as radioactivity and that even between different electrophoretic conditions, intraspecific and interspecific resolution was the same. Silver staining is more time consuming than staining with ethidium bromide but does not present as severe a biohazard. A transilluminator is not needed for the visualization of the gel. All the chemicals for silver staining are kept at room temperature and can be reused. Only a small amount of each specimen is needed for the PCR assay and the test of specimen can be used for other tests such as ELISA assays to determine the sporozoite infection rate. Different methods of DNA preservation can be used depending on the purpose and condition (e.g., dried, frozen in nitrogen, preserved in ethanol or other alcohols) and even museum specimens can be identified. This method is not dependent on the presence or absence of a signal and therefore no false positives are possible.

Hiss et al. (1994) were able to distinguish 8 leafhopper species and 5 different mosquito species with similar taxonomic problems. We suspect that even if

more species of the *An. funestus* group are incorporated in the study, the banding patterns observed will be different from the species tested so far. We did not expect any variation between males and females because rDNA is sex-linked. We did not have access to hybrid specimens and could not test the impact of hybridization on the technique. Banding patterns possibly will be an overlap of the 2 species which took part in hybridization, especially when looking at females.

We conclude that SSCP analysis is a sensitive and reliable method for identification of the 4 members of the *An. funestus* group.

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Chapter 6

ISOENZYME ANALYSIS ON THREE MEMBERS OF THE *AN. FUNESTUS* GROUP

6.1 GENERAL INTRODUCTION TO ISOENZYME ELECTROPHORESIS

Isoenzymes are multiple molecular forms of an enzyme deriving from the same organism, having at least one substrate in common (Brewer, 1970; Harris and Hopkinson, 1976). Isoenzymes catalyse the same reactions but sometimes have different enzyme properties and different kinetics. Isoenzymes result from two or more of the following: 1) multiple gene loci coding structurally distinct polypeptides of the enzyme; 2) multiple alleles at one locus (multiple alleles result in different isoenzyme patterns between individual members of a species) or; 3) by the formation of a secondary isoenzyme due to post-translational modifications of the enzyme structure (Harris and Hopkinson, 1976). The different proteins can be separated by electrophoresis and visualized by histochemical staining of the polyacrylamide gel after electrophoresis. Bands observed after staining are called zymograms (Abderrazak *et al.*, 1993).

6.1.1 History of enzyme electrophoresis

Tiselius is described as the father of electrophoresis with the development of the moving boundary method in 1937 (Brewer, 1970). Moving boundary electrophoresis was followed by zone electrophoresis where proteins were separated in a stabilising medium (agarose, starch, cellulose acetate and acrylamide) rather than in a solution. Agar was first used as a solid medium for the separation of isoenzymes and was gradually replaced with starch and cellulose acetate strips. Today poly-acrylamide is more commonly used as a medium for protein

separation (Brewer, 1970). Histochemical staining techniques are used to visualize isoenzymes.

6.1.2 Principals of isoenzyme electrophoresis

Isoenzyme electrophoresis is a technique where protein molecules in a gel matrix are subjected to an electrical field (Brewer, 1970). Migration of proteins within the electrical field depends on the global electrical charge of the molecule, which is determined by the amino acids comprising the molecule (Abderrazak *et al.*, 1993). A protein molecule has a neutral charge at a specific pH (isoelectrical point or pI), where the molecule has the same number of charged carboxyl and amino groups. At a pH below this point, the amino groups progressively ionize and the molecule assumes a positive charge. An increase in the pH above the isoelectrical point will lead to the progressive ionizing of the carboxyl groups giving a negatively charged molecule. Depending on the number of amino- or carboxyl groups exposed at a specific pH, the molecule will have a specific net electrical charge. The greater the charge on the molecule the faster the migration speed will be towards the opposite electrode.

A second parameter affecting the migration of a protein through a solid medium is the size and shape of the molecule and whether the protein is a multimeric or a monomeric molecule. Multimeric proteins are composed of two or more polypeptide chains or subunits. These subunits may be identical (homomeric isoenzyme) or different (heteromeric isoenzyme) (Harris and Hopkinson, 1976). The banding patterns that show heterozygotes and homozygotes can be used to determine the number of subunits comprising that specific isoenzyme (Harris and Hopkinson, 1976).

Apart from the physical characters of the protein itself, manipulation of the electrophoretic

conditions, such as buffer strength and gel concentration, can help in additional separation of the various enzyme molecules. The strength of the buffer will affect the separation of the protein molecules: the higher the strength, the slower the migration of the molecules. More heat is generated giving good separation of molecules. A change in the electrophoretic conditions will result in a great deal of heterogeneity within electromorphic classes (Ramshaw *et al.*, 1979).

Genetically, isoenzymes behave as alleles. They represent the activity of a genetic locus and once an enzyme is linked to an allele it is referred to as an allozyme or alloenzyme (Abderrazak *et al.*, 1993). To determine whether isoenzyme formations are the product of two different loci or two different alleles, additional information is needed on particular isoenzymes. Experiments such as *in vitro* dissociation and recombination experiments can be performed to answer these questions. Different individuals showing the same zymogram for a specific enzyme are generally an indication that the gene coding for that enzyme does not differ (Bullini, 1984). However, circumstances exist where distinct alleles have enzymes with the same electrophoretic mobility, and this can be determined by other biochemical methods, such as thermostability or enzyme activity.

Specific staining methods can be used to visualize isoenzyme molecules after electrophoresis. These staining solutions contain specific substrates for each enzyme and can be classified as either chromogenic, fluorogenic, radioactive, chemical detection or electron transfer dye (Abderrazak *et al.*, 1993). In certain circumstances a zymogram can be absent which is an indication of a null allele. Null alleles are generally considered to code for non functional allozymes and a homozygote for null alleles will therefore result in no zymogram. Heterozygote

alleles with a null allele will result in the presence of only one zymogram (Bullini, 1984). Homozygote null- allele individuals seem to be viable genotypes with no lethal effects (Tabachnick, 1978).

It is essential to know and understand the basic principles needed to manipulate conditions so as to gain the most value from this powerful technique. Enzyme electrophoresis can be considered as a simple, inexpensive way to examine genetic variability within and between species (Abderrazak *et al.*, 1993). The electrophoresis technique has been employed by various people to differentiate between different insect populations and different species within species complexes (Igbokwe and Downe, 1978). The next section deals with some applications of isoenzyme analysis.

6.1.3 Applications of isoenzyme electrophoresis

Calculating the allele frequency of loci is essential in order to compare different populations or taxa with regards to gene flow, genetic variability, genetic differentiation and estimation of genetic divergence. Isoenzyme electrophoresis has successfully been used in the identification of different members of a sibling species complex as well as providing markers for linkage experiments and for assigning particular gene loci to specific chromosomes or regions of chromosomes (Bullini, 1984). If correctly analysed, isoenzyme electrophoresis provides a valuable tool in population genetics (Abderrazak *et al.*, 1993).

In mosquitoes, electrophoretic markers have been used to construct genetic maps, track mark-recapture experiments and study reproductive behaviour (multiple insemination, mating propensities, etc.). Data collected from such investigations is considered especially useful in

view of the present attempts at genetic control (Bullini and Coluzzi, 1973; Crampton *et al.*, 1994). Electrophoresis has been used in the recognition of species complexes in various mosquito populations (Ayala *et al.*, 1972).

The realization that *An. gambiae s.l.* was a species complex brought about the need to have additional identification methods that were more reliable than morphology. Apart from polytene chromosome studies, isoenzyme electrophoresis was also investigated as a tool for identifying members of the *An. gambiae* complex. Mahon *et al.* (1976) was the first to investigate isoenzyme electrophoresis as a diagnostic system to identify four members of the *An. gambiae* complex in southern Africa. Subsequent studies showed that identification of members of the *An. gambiae* complex could be made in East Africa with an accuracy of >99% (Mahon *et al.*, 1976; Miles, 1978, 1979; Hunt and Coetzee, 1986). However, if the population genotype is unknown, isoenzyme results should be correlated with another method, e.g. chromosome identification. During 1979 Miles published a Biochemical key that can be used to identify six members of the *An. gambiae* complex.

Isoenzyme electrophoresis has the advantage over the chromosome technique in that identification can be made despite sex and stage of the gonotrophic cycle (Miles, 1978). Another advantage is the fact that one can ascertain the genotype of isoenzyme loci, making isoenzymes ideal for population genetic studies, since a co-dominant marker is essential in these studies. Isoenzymes show Mendelian inheritance, which does not complicate population genetic studies (Abderrazak *et al.*, 1993).

Apart from the *An. gambiae* complex, isoenzyme analyses have also been used for

identification of various other mosquito species such as *Aedes triseriatus*, *Ae. hendersoni*, *Ae. aegypti*, *Ae. stimulans* and *Culex* species identification (Scott and McClelland, 1975; Saul *et al.*, 1977; Bullini, 1984; Eldridge *et al.*, 1986).

Green (1977) found sex-limited esterases present in the accessory glands of *An. funestus* males. Apart from this, no other enzyme work has been published on the *An funestus* group. The aim of this study was to investigate different enzyme systems in three members of the *Anopheles funestus* group: *An. vaneedeni*, *An. funestus* and *An. rivulorum*. These data will provide an indication of variability at the intra- and inter-specific levels.

6.2 MATERIALS AND METHODS

Isoenzyme electrophoresis and the staining procedures were based on a protocol provided by L.E. Munstermann (pers. comm.). A detailed description of materials and procedures used as well as E.C. numbers of each enzyme system are provided in Appendix III. Electrophoresis was performed using constant amperage (40mA) and human haemoglobin (hb) used as a marker for enzyme migration. Electrophoresis was generally halted once the haemoglobin had migrated a distance of 2cm from the origin. Mosquito samples were homogenized in 30µl of buffer (10% sucrose, 1:3 dilution of tank buffer, bromophenol blue for colour). 1-2µl of homogenate was loaded per well using a Hamilton syringe. Electrophoresis was followed by staining as described in Appendix III. Electrophoresis conditions tested are summarized in Table 6.1. See Appendix III for enzyme names and abbreviations. Specimens used were from the same localities as those described in Chapter 5 for SSCP electrophoresis.

Table 6.1 Enzyme systems and electrophoresis conditions used in this study. Sample size includes specimens of all three species tested. *Anopheles vaneedeni* and *An. funestus* were generally in equal numbers, but due to limited availability of *An. rivulorum* fewer specimens were included per gel.

ENZYME	TC BUFFER	TBE BUFFER	33: 1	19:1	% GEL	Sample size
AAT	no	yes	yes	yes	6%	18
ACP	yes	no	no	yes	6%	23
AK	yes	yes	yes	no	5%, 7.5%	46
ALP	yes	no	no	yes	6%	23
AO	yes	yes	yes	yes	5, 7.5, 6%	100
DIA	yes	no	yes	no	7.5%	23
EST	no	yes	yes	yes	7.5%	126
GAPD	yes	no	no	yes	5%	23
GCD	no	yes	yes	yes	7.5%	12
GDH	yes	yes	no	yes	7.5%	14
GOT	yes	no	no	yes	6%	13
GPD	Yes	no	no	yes	6%	46
GPI	yes	yes	no	yes	6%, 7.5%	81
GSR	no	yes	no	yes	7.5%	13
G6PDH	yes	no	no	yes	6%	23
HAD	yes	yes	no	yes	7.5%	58
HK	yes	yes	no	yes	5,6%, 7.5%	57
IDH	yes	no	no	yes	5%	23
LAP	yes	no	no	yes	6%	23
LDH	yes	no	no	yes	7.5%	23
MDH	yes	no	no	yes	7.5%	23
ME	yes	yes	yes	no	5%, 7.5%	65
MPI	no	yes	yes	yes	7.5%	284
ODH/SOD	no	yes	no	yes	7.5%	32
PGD	yes	yes	no	yes	7.5%	52
PGM	yes	yes	no	yes	7.5%	52
TREHALOSE	yes	no	no	yes	7.5%	30
XDH	yes	yes	no	yes	7.5%	23

6.3 RESULTS

Twenty-eight different enzyme systems were investigated using two buffers, TBE at pH 8.6 and TC buffer at pH 7.1 (Table 6.1). All enzyme systems were initially standardized on a few *An. funestus* specimens. Once staining and electrophoresis conditions provided good