SYSTEMATIC STUDIES ON THE *ANOPHELES FUNESTUS* (DIPTERA: CULICIDAE) GROUP IN SOUTHERN AFRICA

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DECLARATION

I, L. L. KOEKEMOER declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Koekemoer

29 day of June 1999

This thesis is dedicated to my loving husband, Jan. Thank you for all the love and support you gave me throughout this study and writing of this thesis.

Publications and Presentations Arising From this Study

Publications:

Koekemoer, L.L., Coetzee, M. and R.H. Hunt. 1998. HpaII endonuclease distinguishes between two species in the *Anopheles funestus* group. *Insect Molecular Biology* 7: 237-277.

Koekemoer, L.L., Hunt, R.H. and M. Coetzee. 1999. Single Strand Conformation Polymorphism analysis for identification of four members of the *Anopheles funestus* group (Diptera: Culicidae). *Journal of Medical Entomology* 36: 124-130.

Presentations:

Koekemoer, L.L. 1997. Molecular techniques to identify members of the *Anopheles funestus* group. *Proceedings of the Joint Congress of the Entomological Society of Southern Africa (11th congress) and the African Association of Insect Scientists (12th congress)*, Stellenbosch, 30 June -4 July.

Koekemoer, L.L., Coetzee, M. and R.H. Hunt 1998. Molecular identification of four members in the *Anopheles funestus* (Diptera:Culicidae) group using Single-Strand Conformation Polymorphism. *2nd European Congress on Tropical Medicine of the Federation of European Societies for Tropical Medicine and International Health and 4th Residential Meeting of the Royal Society of Tropical Medicine and Hygiene. September 14-18, 1998 Liverpool, UK*, page141.

Koekemoer,L.L., M. Coetzee and R.H.Hunt. 1998. Genetic studies on the *Anopheles funestus* Giles group (Culicidae) in southern Africa. *Fourth International Congress of Dipterology, 6-13 September 1998 Keble College, Oxford, UK: page* 108.

Koekemoer, L.L. 1999 Molecular identification of four members in the *Anopheles funestus* (Diptera:Culicidae) group using Single-Strand Conformation Polymorphism. *MIM African Malaria Conference. 14-19 March. Durban, South Africa.*

Koekemoer, L.L. and J.P. La Grange. 1999. Sporozoite infections in female Anopheline mosquitoes from Mpumalanga. *MIM African Malaria Conference. 14-19 March. Durban, South Africa.*

ABSTRACT

Members of the *An. funestus* group occur abundantly in malarious areas in Africa, and are difficult to identify morphologically. Identification using polytene chromosomes is limited to half gravid female mosquitoes. This study tried to establish the role of the *Anopheles funestus* group in malaria transmission and aimed to develop a molecular method for rapid identification of members of the *An. funestus* group. Wild caught female *An. funestus* group were collected at various localities in Africa. Morphological and polytene chromosome identifications were performed. Identified specimens were analysed to locate variable regions of the rDNA for the development of a species-specific PCR assay. A PCR-Single Strand Conformation Polymorphism (SSCP) was able to distinguish between four members of the group, *An. funestus*, *An. vaneedeni*, *An. rivulorum* and *An. leesoni*. The PCR-SSCP assay appears to be a valuable tool for the identification of members of the *An. funestus* group and will prove useful to malaria control programme managers for their mosquito surveillance activities.

Isoenzyme analysis was conducted on fresh and frozen specimens to establish if isoenzyme systems can be used for either identification or population genetic studies. Isoenzyme studies revealed nine diagnostic systems as well as thirteen systems that can potentially be used in population genetic studies.

Morphologically identified *An. funestus* group from Mpumalanga province, South Africa, were sent to the SAIMR to establish malaria parasite infection rate in these specimens and to identify infected species once a molecular technique was established. ELISA analysis revealed an abnormally high infection rate of *P. falciparum* in specimens collected during April 1997. A PCR assay for detecting *P. falciparum* DNA was used to confirm these results. The majority

of specimens were negative on PCR analysis, indicating the absence of *P. falciparum* DNA. PCR-SSCP analysis on positive-ELISA specimens revealed abnormal banding patterns, not diagnostic for members of the *An. funestus* group. Morphological analysis performed on the remains of wings identified them as *An. demeilloni* and member (s) of the *An. marshallii* group.

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INTRODUCTION

1.1 GENERAL INTRODUCTION

Insects are responsible for transmitting a wide variety of pathogenic organisms to humans. This is particularly true in tropical regions where these diseases cause major health problems. Parasitic diseases such as malaria, filariasis, leishmaniasis, river blindness and various viral diseases such as yellow fever are transmitted by insects. The most important of these is malaria, which, despite enormous efforts over many years, is an increasingly important health problem with more than 40% of the world population exposed to malaria (Crampton *et al.*, 1994). Recent estimates indicate that malaria is endemic in 102 countries worldwide and 2 billon people, representing almost half the world's population, are at risk from the disease. There are an estimated 200 million malaria infections and 1-3 million deaths annually (World Health Report, 1996). This one disease has an enormous impact on the health and economies of many tropical countries (Strürchler, 1989; Collins and Paskewitz, 1995; World Health Report, 1996).

Malaria is caused by infection with the protozoan parasite of the genus *Plasmodium*. The most severe malaria is caused by the predominant species in tropical Africa, *Plasmodium falciparum*. The main mode of transmission is exclusively by a few species of mosquitoes within the genus *Anopheles* (Gillies, 1988). Prior to the 1950s malaria was mainly controlled by prevention of contact between mosquitoes and humans, eg. mosquito bednets and the screening of houses (Gillies, 1988). Larviciding in the first half of the century was also popular as a means of reducing mosquito numbers. These measures, however were not very successful (Hooey,

1974). G. Giemsa used pyrethrum to kill culicine mosquitoes in German cellars during 1910 and Ingram and De Meillon found the same situation in their experiments with houseflies (as recorded in Coetzee and Hunt, 1993). During 1936 De Meillon submitted a report to the Pan-African Health Conference of the League of Nations, covering the studies that were carried out in Natal to control adult mosquitoes in houses using pyrethrum spraying. A similar study was conducted in India using De Meillon's idea and was most successful (Coetzee and Hunt, 1993). The work done by De Meillon laid the foundation for the World Health Organization's 1950's campaign to eradicate malaria world wide.

The history of malaria and its transmission dates back to the end of the nineteenth century when it was discovered that *Anopheles* mosquitoes were transmitting malaria parasites to humans. Twenty percent of the almost 500 *Anopheles* species described have been implicated as vectors, but most are minor or incidental vectors (Gillies, 1988). In sub-Saharan Africa, the main vectors involved in transmission are two species of the *Anopheles gambiae* complex, *An. gambiae* Giles and *An. arabiensis* Patton, and a third species, *An. funestus* Giles. *Anopheles gambiae* and *An. funestus* are both highly anthropophilic and endophilic preferring to feed on humans and rest in human habitations (Gillies and De Meillon, 1968). *Anopheles arabiensis*, on the other hand, is both zoophilic and anthropophilic and will rest indoors or outdoors. In certain areas of Tanzania it has been shown that another species of the *An. gambiae* complex, *An. merus*, is as good a vector of malaria as the three mentioned above (Temu *et al.*, 1998).

1.2 VECTOR CONTROL - CURRENT AND NEW APPROACHES

One of the most important problems in malaria control is the difficulty of controlling transmission of the parasite. In most African countries the use of prophylactic medicine is too

expensive and cannot be prescribed for long periods of time. The second problem associated with parasite control is the presence of widespread drug resistance. Considerable variation in malaria epidemiology exists (even within a country) due to geography, ecology and human activities, which complicates vector control programmes since no single control tool or approach can be applied (World Health Statistics Quarterly, 1992).

For effective vector control it is essential to understand all the aspects involved such as the malaria incidence, risk factors, vector/ human host contact and environmental conditions. Vector control can be achieved with varying success by indoor spraying of houses with residual insecticides, personal protection such as insecticide treated bed nets, biological control, larviciding and environmental management (Coluzzi, 1992).

The aim of vector control is to reduce or eliminate the vector population. This can be done by reducing the adult population, reducing the life span of the adult female and by the prevention of contact between humans and vectors (Coluzzi, 1992). Once vector control has been implemented it is important to determine what the effect of vector control is and whether it is effective or not. It is important to monitor the insecticide resistance in the area and to monitor the presence of plasmodial sporozoites in salivary glands of malaria vectors. The presence of sporozoites is also an indication of the vector status of that specific species in which the sporozoites were found (Beier *et al.*, 1991). The sporozoite rate can also give information regarding the estimated inoculation rate of human biting mosquitoes, which is a key factor in quantitative analysis of natural transmission.

1.2.1 Future prospects for vector control

Modifying insects rather than eradicating them is an alternative method of control, since eradication with harmful chemicals is not recommended in a complex ecological network. Chemical eradication of insects leads to the occurrence of resistance, concern over environmental issues and is an extremely expensive method to maintain continuously (Beard *et al.*, 1993a). Genetically incompetent mosquitoes, unable to transmit the parasite, would be expensive to develop, but would be cheaper to maintain in the long run.

The search for a single 'magic' control tool against malaria has been, and will probably continue to be, the driving force for many scientists involved in malaria vector research. The introduction of molecular biology has opened up a new field of research aimed at genetically transforming vector mosquitoes into non-vectors. The advantages of genetic engineering lie in the potential to exploit genes across species barriers and in the ability to introduce defined DNA sequences without the genome disruption that accompanies conventional selection and crossing procedures (Coluzzi, 1992).

Naturally occurring refractoriness to *Plasmodium* parasites has been recorded over the years in several mosquito species, but does not seem to have a selective advantage for the vector. The aim of researchers is to isolate these genes, study them, and using the knowledge gained, genetically engineer a vector and spread the engineered refractoriness through the natural population (Beard *et al.*, 1993a). This refractoriness, however, is probably a complicated system with multiple genes playing a role in the process. A more realistic way would be to interfere with the pathogen by inserting a gene(s) into the vector which produces anti-pathogenic products.

A transformation vector system will be needed to insert and express these foreign gene(s) that render the vector refractory. Two main transformation vector systems are being investigated, the first being transposons and the second symbiotic bacteria.

1.2.1.1 Transposable elements

Transposable elements may provide a good system for driving the gene or gene complex through a natural population. Transposons are vertically transmissible and have been demonstrated in *Drosophila* (Spradling and Rubin, 1982; Kiszewski and Spielman, 1998). The P-element mediated germline transformation in *Drosophila* but not in other insect species (Beard *et al.*, 1993a). Success has been achieved using this approach in the mosquito *Aedes aegypti*, which was successfully transformed using a modified *Hermes* transposable element (Jasinskiene *et al.*, 1998).

1.2.1.2 Symbiotic bacteria

Symbiotic bacteria on the other hand could be used to introduce and express the product of the foreign gene. The product is then secreted by the bacterial system. Some arthropods commonly carry symbiotic microorganisms to provide additional supplements to the diet (Beard *et al.*, 1993a). This product can be either extracellular (symbiont of *Rhodnius prolixels*) or intra-cellular (*Rickettsia*-like organisms) (Beard *et al.*, 1993a). The transformed symbiont must be maintained through successive generations and must not have a negative or lethal effect on the organism. Bacterial-gene spreading has advantages over mechanisms such as transposons because it can spread genes into the same population more than once. Horizontal transmission occurs naturally (Beard *et al.*, 1993a).

Transgenic technology has the advantage of crossing species barriers, with specific DNA being introduced into the genome. In conventional crosses the genome is disrupted with lethal consequences (Warren and Crampton, 1994). If the introgression hypothesis between *An. gambiae* and *An. arabiensis* is shown to be correct (Besansky *et al.*, 1997) it may play a major role in the introduction of transgenic mosquitoes for malaria control. Whatever the outcome of this research may be, the exclusion of the third major African vector *An. funestus* from these studies could yet prove to be the downfall of the system as an effective control tool.

1.3 SPECIES CONCEPTS

The morphological differences observed between populations are not always an indication that two species are present. Likewise, morphological differentiation need not accompany speciation events. It is appropriate, therefore, to examine the concepts surrounding speciation and what defines a species.

Vrba (1995) describes four species concepts in relation to complex systems. The first concept is the phylogenetic concept which requires that species are strictly monophyletic. This concept is based clearly on nested homologies at the level of organisms and their constituents. Templeton (1989) tried to include asexual as well as sexual forms into a species concept called the Cohesion Concept. Templeton defined cohesion as genetically based phenotypic cohesion. The other two species concepts (the biological and recognition species concepts) will be discussed in more detail below. The various ideas on species concepts are so intricately complicated that to summarize them in a single chapter is extremely difficult. The basic differences between these four species concepts are summarized in Table 1.1 (Adapted from Vrba, 1995).

Table 1.1 Required character divergence for the four species concepts

	Required character divergence						
Species concept	**Pre-fertilization system**		**Post-fertilization system**		**Habitat adaptation**		**Any other**
Recognition	Yes						
Isolation	Yes	or	Yes				
Cohesion	Yes	or	Yes	or	Yes		
Phylogenetic	Yes	or	Yes	or	Yes	or	Yes

1.3.1 Biological species concept: The Isolation concept

This species concept was mainly defined by Dobzhansky during 1935 and 1937 and Mayr (1942, 1963). Mayr (1942) introduced the term sibling species and defined them as "morphologically similar and identical natural populations that are reproductively isolated". Reproductive isolation is caused by pre- and/ or post-fertilization mechanisms.

Pre-fertilization and pre-mating isolating mechanisms can be due to ecological isolation. In ecological isolation the potential mates do not meet because they mate in different habitats or in different seasons. Ethological isolation is defined as where potential mates meet, but do not mate. Post-mating isolation, before fertilization takes place, happens when there is a mechanical isolating mechanism such as no sperm transfer after copulation. Another example of post-mating isolation is gametic mortality or incompatibility where the egg is never fertilized even though sperm transfer occurs.

Post-fertilization (post-zygotic) isolation occurs when the egg was successfully fertilized. Four modes of isolation are grouped in this class. Firstly, F_1 inviability, occurring because the zygote

dies or produces a hybrid of reduced viability. Secondly where the F_1 is viable but sterile either completely or partially. Thirdly, when the F_2 or backcross hybrid breaks down due to reduced viability or fertility. Fourthly, where the endoparasitic or cytoplasmic infection of a population results in fertility or viability breakdown after mating with uninfected individuals, even though the infected individuals are infertile (Mayr, 1963).

Sibling species are generally discovered due to variation observed in habitats, host preferences, vector status and so forth. In the *An. gambiae* complex sibling species were recognized by hybridization tests and differences in karyotypes (Davidson, 1962; Paterson, 1962; Davidson, *et al.*, 1967).

Within the biological species concept, natural selection also plays an important role in the isolation of two species. The biological species concept however, does not explain how *ad hoc* isolation mechanisms (adaptations) arise in an allopatric situation (Macnamara and Paterson, 1984). Despite this, natural selection is supposed to reinforce incipient isolation of populations living in sympatry. Natural selection shifts the genetic composition of a population in response to environmental demands (Carson, 1976) and this can then be seen in the phylogenetic analysis of that population.

1.3.2 The Recognition species concept

This species concept defines a species as that "most inclusive population of individual, biparental organisms which share a common fertilization system in their preferred habitat" (Paterson, 1985). It is these fertilization mechanisms which determine the limit of the species gene pool (Macnamara and Paterson, 1984). The pre-fertilization system is the focus of the