recognition concept (Vrba, 1995).

Paterson identified two facts misrepresenting the isolation concept, firstly the fact that allopatric speciation is the only empirically supported mode of speciation and secondly, that allopatric speciation cannot account for the origin of isolating mechanisms (Macnamara and Paterson, 1984). In Paterson's recognition concept the specific-mate recognition system (SMRS) is the key hypothesis and using this, a species can be defined as populations of individual organisms which share a common SMRS (Paterson, 1986). According to this idea species are thus incidental effects of adaptive evolution (Paterson, 1993). The present study was carried out using the Recognition concept as the theoretical basis for species delimitation.

1.4 THE *Anopheles funestus* GROUP

This group consists of eight recognised species in the Afrotropical region. They are characterized as small, dark mosquitoes showing great uniformity in adult morphology and are placed in the Series Myzomyia by Gillies and De Meillon (1968). Green (1982) performed phylogenetic studies on the Myzomyia series using inversion rearrangements found on the giant polytene chromosomes. Green found that within the Myzomyia series four groups can be defined. The first group, "*funestus* group" consists of *An. funestus*, *An. vaneedeni*, *An. parensis* and *An. confusus*. The second group, "*rivulorum* group" contains *An. rivulorum* and *An. fuscivenosus*. The "*leesoni* group" contains *An. leesoni* and two Asian species *An. fluviatilis* and *An. culicifacies*, and the fourth group, the "*wellcomei* group" has *An. theileri* and *An. demeilloni* as members. Green agreed with the decision of Gillies and De Meillon (1968) to recognise *An. confusus* and *An. fuscivenosus* as good species. However, his data suggest that *An. fluviatilis* and *An. leesoni* are not members of the *An. funestus* group, although the sample sizes were very small (*An. fluviatilis* n=2, *An. leesoni* n=24). For the writing of this thesis I will continue to consider *An. leesoni* as part of the *An. funestus* group because of the similarity in adult morphology.

1.4.1 A historical overview of the *An. funestus* group

Anopheles funestus was first described by Giles in 1900. Thirty years later Evans and Leeson recorded the existence of distinct "varieties" (Leeson, 1930a; Evans, 1930, 1931). During a study in Zimbabwe (Leeson, 1930a) specimens of *An. funestus* were grouped into 10 distinct groups based on the arrangements of scales on the costa as well as the first longitudinal vein. Nine distinct arrangements of wing scales were observed. Leeson (1930b) described a new "variant" of *An. funestus* that was caught outdoors in the dry season and named it *An. funestus* var. *fuscivenosus*. Evans (1930) described distinct characteristics of *An. funestus* compared with the oriental species *An. minimus* and *An. histoni*. Symes (1931) described the larvae of *An. funestus* and in 1931 Evans described the *An. funestus* subspecies *leesoni*, from southern Rhodesia (Zimbabwe). Evans and Garnham (1936) described the differences between *An. funestus* and *An. rivulorum*. Leeson (1937) found that it was easier to identify the larvae than the adults in members of the *An. funestus* group.

In 1960 Service (1960a) observed two different strains of *An. funestus* in southern and northern Nigeria. The strain from southern Nigeria was more susceptible to insecticides than the strain from northern Nigeria. Gillies (1962) described *An. funestus* var. *confusus* Evans and Leeson which is restricted to East and southern Africa. In 1964 there were four accepted species within the *An. funestus* group, *An. funestus*, *An. leesoni*, *An. parensis* and *An. rivulorum* (Service, 1964), although Gillies (1962) raised *An. funestus* var. *confusus* to species rank. Gillies and De Meillon (1968) introduced the term "*funestus* subgroup" and recognised eight species within this subgroup.

1.4.2 Members of the *An. funestus* group, their biology, vector status and distribution

a) *An. aruni* Sobti 1968

Anopheles aruni was described from Kaburi Kikombe, Zanzibar. Female *An. aruni* bite humans outside at night but apart from this, little is known about the adult behaviour, vector status or biology of this species.

b) *An. brucei* Service 1960(b)

Anopheles brucei was recorded from Lokoja in northern Nigeria. Larval habitats have been recorded as shady forest streams and partially dried riverbeds (Gillies and De Meillon, 1968). Limited information is available on the biology of this species.

c) *An. confusus* Evans and Leeson 1935

The type locality for *An. confusus* is recorded as Umtali and Sinoia, Rhodesia (Zimbabwe) (Gillies and De Meillon, 1968). Larvae are generally found in slow flowing water, but White (1972) reported to have found larva breeding in hippopotamus footprints in Ethiopia. Adults can be found inside houses on occasion. *Anopheles confusus* occurs in the plateau areas of eastern Africa stretching from Kenya and Ethiopia to the northern and eastern parts of South Africa and in Zimbabwe (Gillies and De Meillon, 1968). It is not well described and its biology and vector status is unclear.

d) *An. funestus s.s.* Giles 1900

Anopheles funestus is the most dominant member of the *An. funestus* group with regards to numbers and distribution and occurs in most parts of tropical Africa (Gillies and De Meillon, 1968). The biology of *An. funestus* is well studied with extensive information available on larval and adult biology (Gillies and De Meillon, 1968). Larvae of *An. funestus*, like other members in the group, occur wherever sufficient permanent water bodies are available e.g. swamps, weedy sides of streams, rivers and even in wells and domestic containers (Symes, 1936; Leeson, 1937). *Anopheles funestus* males have not been found to swarm, not even in areas with a high density of mosquitoes (Gillies and De Meillon, 1968). The only record of swarming was observed by Harper (1944) in Kenya, where it was found during dusk at one to two feet above the veranda of a house. Sixty eight specimens were captured by Harper and all were identified as *An. funestus* males using a hand lens. *Anopheles funestus* disperses over short distances and generally no more than one mile (1.61 km), but when food and oviposition sites are spread, dispersing will occur over larger areas (Gillies and De Meillon, 1968).

Anopheles funestus mainly feeds in the second half of the night, but has been recorded biting until the early hours of the morning and will feed during the day in dark houses (Gillies and De Meillon, 1968). After blood feeding the females tend to stay indoors until they are gravid (2-3 days) before leaving the houses (Gillies and De Meillon, 1968). *Anopheles funestus* is the most anthropophilic member of the group and even in the presence of ample animals, will still seek humans as a host. It is only in circumstances where cattle mask the presence of humans that *An. funestus* will feed on animals (4-8%) (Gillies and De Meillon, 1968). The fact that the adult *An. funestus* spends most of its life inside houses makes it extremely susceptible to control by residual insecticides.

The infection rates of *P. falciparum* recorded for *An. funestus* are usually lower than *An. gambiae* infections in the same region and are generally between 2-5% (Gillies and De Meillon, 1968). Salivary gland dissection performed on *An. funestus* from Senegal showed infection rates of *P. falciparum* between 1.35 - 6.45% in five localities over five years (1992-1997) (Lochouarn *et al.*, 1998). Studies done in Bagamoya (coastal Tanzania) showed that at specific times of the year, *An. funestus* can have a higher infection rate than either *An. gambiae* or *An. arabiensis* (Temu *et al.*, 1998). Besides malaria, *An. funestus* also transmits other infectious pathogens such as Bancroftian filariasis and Tanga virus. *Anopheles funestus* females were found to be infected (0.5%) with the larvae of *Wuchereria bancrofti* in Burkina Faso (Gillies and Coetzee, 1987). Gillies and Coetzee, (1987) summarised other viruses isolated from *An. funestus* (Table 1.2).

Anopheles funestus is considered eurygamic and refractory to colonization (Gillies and De Meillon, 1968).

Table 1.2 Virus isolation from *An. funestus* (Gillies and Coetzee, 1987).

Jos plateau, Nigeria	Bwanba Bunyamwere
Western Ethiopia	Tataguine
Uganda	Orunga
Kenya	Akabana Germiston virus near Tataguine
East Africa	Hepatitis B antigen

e) *An. fuscivenosus* Leeson 1930(b)

Anopheles fuscivenosus is only known in the adult stage with the type locality recorded as Shamva, Rhodesia (Zimbabwe). The adults resemble *An. funestus* and were collected in outdoor resting-sites. The early stages of the life cycle is unknown (Gillies and De Meillon, 1968).

f) *An. leesoni* Evans 1931

Anopheles leesoni type locality is recorded as Rhodesia (Zimbabwe) with no mention of precise location. *Anopheles leesoni* has also been found in the Savannah regions of Eastern and West Africa and occurs in Ethiopia, Kenya, Uganda through to Mozambique and in South Africa and from Côte d'Ivoire to northern Nigeria (Gillies and De Meillon, 1968). Larval habitats are generally the same as for *An. funestus* (Gillies and De Meillon, 1968). Adults are caught inside houses on rare occasions, but are more commonly collected in natural resting sites and are mainly zoophilic. De Meillon (1935) dissected *An. leesoni* salivary glands to determine if this species was a possible vector of malaria. The sample size was very small (n=11) and no infective salivary glands were found.

g) *An. parensis* Gillies 1962

Anopheles parensis type locality is Kihurio, South Pare district in Tanzania (Gillies and De Meillon, 1968). Early life stages resemble *An. funestus* closely and unlike other members of the *An. funestus* group this member seems to be absent from streams and moving water (Gillies and De Meillon, 1968). Gillies and Furlong (1964) collected *An. parensis* in a sprayed zone in coastal Kenya and adults were caught biting humans outdoors at night. *Anopheles parensis* was also collected indoors, but in small numbers (Gillies and De Meillon, 1968). *Anopheles* *parensis* has not been shown to be infected with *P. falciparum* (Gillies and De Meillon, 1968). This species occurs in East Africa from Kenya, north-eastern Tanzania, Swaziland and KwaZulu-Natal, South Africa.

h) *An. rivulorum* Leeson 1935

Anopheles rivulorum was described from Sinoia, Rhodesia (Zimbabwe) (Gillies and De Meillon, 1968). It is the second most widespread member of the group occurring mainly in eastern and southern Africa from Ethiopia through to Zimbabwe, Mozambique, Botswana, and in South Africa. In West Africa it occurs from Mali and Côte d'Ivoire to northern Nigeria (Gillies and De Meillon, 1968).

Evans and Garnham (1936) documented morphological variation among the series *An. rivulorum* from two localities in Kenya, while Evans and Leeson (1937) found the same variation in additional localities from Tanzania and Uganda. Evans and Leeson (1937) studied *'An. rivulorum'* in Tanzania and found that the characters by which the species are distinguished appear unstable and that an intergrading local form exists. *Anopheles rivulorum* var *garnhamellus* has characteristics differing from *Anopheles rivulorum* type form. Figure 1.1 is a diagrammatic description between wing types from above mentioned variants.

An. rivulorum is not influenced by residual house spraying, because it is mainly a exophilic and zoophilic species. The population of *An. rivulorum* increased when *An. funestus* populations decreased (3-7 fold increase) in an area where spaying was carried out for a three year period (Gillies and Smith, 1960). This might be explained by larval competition being reduced (Gillies and Smith, 1960).

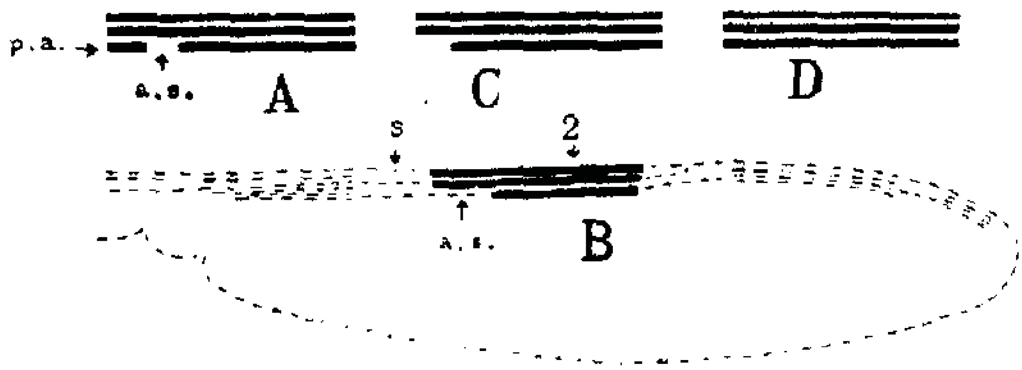

Figure 1.1 Showing wing variation on the second main dark area on the first vein between *An. rivulorum* type form and *An. rivulorum* var. *garnhamellur*. (p.a = pre-accessory sector spot; s= sector spot; a.s.= accessory sector spot). **A**- *An. rivulorum*; **B** and **C**- *An. rivulorum* var. *garnhamellur*, **D**- very rare (Evans and Leeson, 1937).

Gillies and Smith (1960) dissected 66 *An. rivulorum* female mosquitoes and no infections of *Plasmodium* sporozoites were found. Wilkes *et al.* (1996) on the other hand recently showed that *An. rivulorum* in Tanzania was infected with *Plasmodium* sporozoites and so could maintain malaria transmission in the absence of *An. funestus*. *Anopheles rivulorum* will bite humans outdoors in the absence of animals.

i) *An. vaneedeni* Gillies and Coetzee 1987

Anopheles vaneedeni was described from Tzaneen in the Northern Province, South Africa. Larval habitats are recorded as the same as those of *An. funestus*. Female *An. vaneedeni* have been found resting in pit-shelters with other species such as *An. leesoni*. Studies on adult behaviour showed that it will readily bite humans outside in the early hours of the night but will not feed or rest indoors (De Meillon *et al.*, 1977).

To determine if this species was capable of transmitting malaria, 48 females were experimentally fed on a gametocyte carrier. Five out of 28 (17%) dissected were positive for oocysts on the stomach walls and three out of 14 (21%) were positive for sporozoites in the salivary glands. These studies provide strong evidence that *An. vaneedeni* can transmit *P. falciparum* (De Meillon *et al.*, 1977). It is uncertain, however, what the role of *An. vaneedeni* is in malaria transmission (De Meillon *et al.*, 1977; Smith *et al.*, 1977). The difficulty associated with the identification of *An. vaneedeni* has resulted in the lack of transmission studies to date.

1.4.3 Identification of the species

Currently only two methods can be used to identify members of the *An. funestus* group. Firstly by studying the external morphology of the various life stages, and secondly, through the use of polytene chromosomes. Both these methods have limitations in their use and reliability.

1.4.3.1 Morphological differences between members of the *An. funestus* group

To study the morphology of a specimen all the life stages are essential. This implies that wild females must be caught and brought back to special rearing facilities, blood fed and any eggs produced reared through to the adult stage. Old specimens brought back from the field are difficult to study morphologically, because of scales that have been rubbed off or sometimes physically damaged. Eggs and the pelts of both the fourth instar larvae and the pupa are studied. Newly hatched adults are pinned for future reference. The keys and other illustrated characteristics described in Gillies and De Meillon (1968) and Gillies and Coetzee (1987) are used for identification.

This method is time consuming and it can take six weeks or longer before identifications are completed. Identification of field samples may be limited because of larval mortalities or egg batches that fail to hatch. Another problem is the morphological overlap of diagnostic characters in certain species. Detailed descriptions of the differences occurring between the species at different life stages can be found in Gillies and De Meillon, (1968) and Gillies and Coetzee, (1987). Appendix I contains schematic drawings depicting morphological differences between species in the *An. funestus* group. The following is a short summary from these two references.

a) Adult stages

- *Anopheles confusus* closely resembles *An. funestus* and the females are externally morphologically indistinguishable. The pharynx of female *An. confusus* and the presence of a pale patch of scales on the club of male palps which is present in 50% of specimens can be used in identification of *An. confusus*. The length of the external accessory hairs on the terminalia in East African specimens can be used to distinguish *An. confusus* from *An. funestus*, however these can resemble *An. leesoni* in adult morphology.
- *Anopheles fuscivenosus* differs from *An. funestus* having darker wings with different wing characteristics.
- *Anopheles leesoni* resembles *An. funestus* in adult morphology.
- *Anopheles parensis* males can be separated from *An. funestus* by the presence of a pale band at the base of the club in 70-75% of specimens, but it cannot be separated from *An. brucei* (Gillies and Furlong, 1964).
- *Anopheles brucei* resembles *An. rivulorum*, but can be separated from it by comparing female palps and pharynx, mesonotum and wings. Male palps and terminalia are also

used in separating these two species.

- *Anopheles rivulorum* is quite distinct from other members in that young adults have an orange-brown scutum that is characteristic.
- *Anopheles aruni* females can be distinguished from *An. funestus* by broadly banded palps and paler wings. Plotting wing-spot ratio against palpal band ratio distinguish *An. aruni* females from all the other members.
- *Anopheles vaneedeni* resembles *An. aruni* but can be separated by darker wings, breadth of the pale band occurring on the palps, and a difference in wingtip ratio and palpal band ratio. It can be separated from *An. funestus* using a pre-sector pale spot on the costa in 97% of specimens. Ninety-nine percent of specimens have a pale spot in the middle of vein 3, however 50% of *An. funestus* also have this spot. *Anopheles vaneedeni* also has pale bands at the joints of their tarsomeres, which, when present, are diagnostic.

b) Larval stages

Anopheles leesoni and *An. confusus* can be identified using differences on the larval pelts, such as the clypeal patterns and abdominal plates. *Anopheles brucei* resembles *An. rivulorum* in the larva stages, but can be separated on the length of the clypeal hairs, and by the accessory plates. *Anopheles aruni, An. parensis* and *An. vaneedeni* are indistinguishable from *An. funestus*. The larva of *An. fuscuvenosus* is unknown.

c) Pupal stages

Anopheles confusus is separated from *An. funestus* by seta 1 on the pupa. *Anopheles leesoni* and *An. rivulorum* are separated from *An. funestus* and from other members in the group, but the pupa of *An. brucei* resembles that of *An. rivulorum*. The pupae of *An. aruni*, *An. parensis* and *An. vaneedeni* are not separable from *An. funestus*. The pupa of *An. fuscivenosus* is unknown.

d) Eggs

Anopheles leesoni eggs are the only ones that are distinctly different from all other members of the group (see Appendix 1). The eggs of *An. fuscivenosus* is unknown.

1.4.3.2 Identification using polytene chromosomes

Polytene chromosomes are giant chromosomes with characteristic banding patterns found in many Diptera. In anopheline mosquitoes, polytene chromosomes are found in the salivary glands of the fourth instar larvae, and in the nurse cells of half gravid ovaries of the females. They are stained using propionic acid and lacto-acetic orcein (Hunt, 1973). This method can be used to identify *An. parensis, An. rivulorum, An. leesoni* and *An. confusus* (Green, 1982). *Anopheles vaneedeni* and *An. funestus* are homosequential species, i.e. they have identical banding arrangements, except for a floating inversion which occurs on arm 2, thus limiting the identification of two species (Green and Hunt, 1980). The reading and analysing of the banding patterns needs a great deal of expertise.

1.5 <u>MALARIA SITUATION IN SOUTH AFRICA</u>

Malaria transmission in South Africa is generally seasonal, unstable and confined to the low-lying areas of the three provinces. The Ingwavuma district in KwaZulu/Natal Province is a high risk area, while the intermediate risk area stretches from Kosi Bay in the north to Lake St Lucia in the south. During 1996 there were approximately 10 535 reported malaria cases with 32 deaths in the KwaZulu/Natal province (Malaria Report, 1998a). These figures increased during 1997 when 11 425 cases were reported and 38 people died from malaria infections. The Northern Province has fewer reported malaria cases and during 1996, 5 211 cases were reported by the Department of Health. Sixty five people died during the 1996 calender year. The reported malaria cases increased during 1997 when 4 649 cases were reported and 44 people died due to infection (Malaria Report, 1998b).

The local population in Mpumalanga Province have a low protective immunity and the majority of infections are considered to have their origin in Mozambique (Malaria Report, 1998c). The Department of Health reported 10 192 malaria cases in Mpumalanga during 1996 resulting in 58 deaths. During 1997, 5 787 malaria cases were reported with 11 deaths. Figure 1.2 is a diagrammatic illustration of the increase of malaria infections in South Africa. Deltamethrin is used as the insecticide of choice for spraying houses and temephos is used for limited larval control (Malaria Report, 1998b; 1998c). No insecticide resistance has been reported from any of the malarious areas.

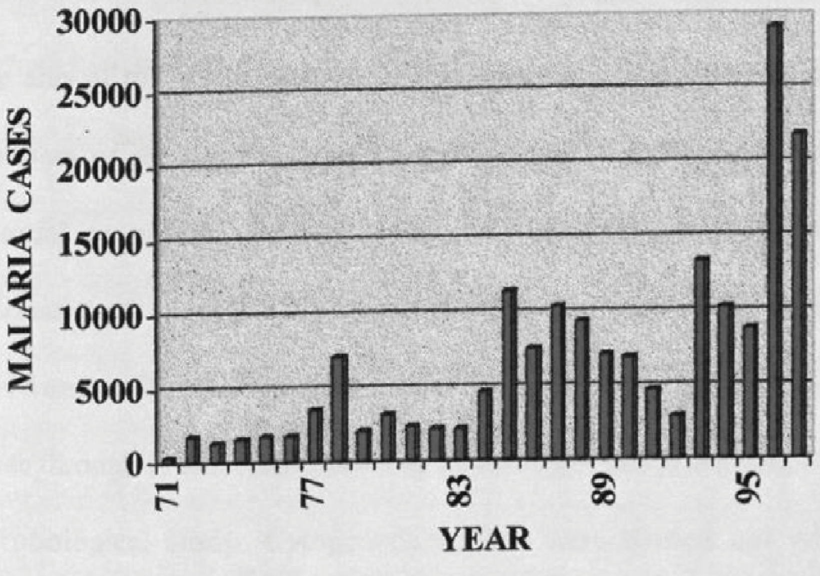

Figure 1.2 Depicting malaria cases in South Africa during 1971 to 1997.

1.6 OBJECTIVES OF THE STUDY

In the light of continued malaria transmission despite effective vector control programmes, it is essential to determine the vector specie responsible for maintaining the transmission in malarious areas. While it is possible to identify members of the *An. gambiae* complex at this stage it is not possible to identify the members of the *An. funestus* group once they have been dried and tested for parasite infections by ELISA assays, because no molecular assay exists.

At least three members of the *An. funestus* group are found in Mpumalanga, and are caught in large numbers biting humans outside at night. Preliminary investigations showed 0.3% of the *An. funestus* group collected in Mpumalanga, were positive for circumsporozoite proteins of *P. falciparum*. Unfortunately, the species could not be identified. This created a problem since the malaria control programme manager could not adapt the control programme to target these species specifically and is one of the reasons why this project was initiated. Furthermore, little work has been done on this group because of the difficulty in maintaining and rearing these mosquitoes under standard insectary conditions.

The aim of the study was to develop a molecular assay to distinguish the most common members of the *An. funestus* group occurring in South Africa. To achieve this aim it was essential to collect members of the *An. funestus* group, gain as much morphological information on all the life stages and correctly identify these specimens. This meant collecting eggs from a wild caught female mosquito, recording the morphology of the eggs, rearing the larvae through to adult mosquitoes in the insectary and preserving larvae, pupae and adults for morphological study. Cytogenetic studies were carried out where possible to assist in identifying the species correctly.

The development of the molecular assay was based on the same principles that exist for the *An. gambiae* complex where similar identification problems were experienced. This involved sequencing of specific DNA regions for detecting species-specific variation, which can be used to develop species-specific primers. Using a PCR assay has enormous advantages. Only a small portion of a mosquito is needed for identifying the specimen and the remaining parts of the mosquito can be used for transmission studies. Preservation of samples in the field is uncomplicated and specimens can be stored on desiccant or in alcohol.

The investigation of various isoenzyme systems was carried out as well, since no recorded data are available on enzymatic variation in the *An. funestus* group. The aim was to find systems that are diagnostic for members of the group.

Chapter 2

COLLECTION OF WILD CAUGHT MATERIAL AND *A PRIORI* IDENTIFICATION

2.1 INTRODUCTION

In order to achieve the objectives set out for this thesis it was essential to have accurately identified specimens to be used as reference material in both DNA and protein studies. Owing to the morphological overlap between members of the *An. funestus* group, discussed in Chapter 1, it was necessary to study the morphology of all life stages. This, however, is very time consuming and the period from egg to adult can take between 4-6 weeks (Gillies and De Meillon, 1968). This is probably one of the major reasons why *An. funestus* has never been colonized successfully for any length of time. Service and Oguamah (1958) managed to colonize *An. funestus* in Lagos for a number of months, but apart from this, no literature is available on colonization and rearing *An. funestus* under insectary conditions.

Part of this project included investigation of various rearing possibilities in order to increase viability of progeny under insectary conditions.

2.2 MATERIALS AND METHODS

Field collections:

The samples used throughout this study originated from the localities summarized in Table 2.1

Table 2.1 Specimens used in this study with the locality, collection method and date

Country	Locality	Collection method	Date
Madagascar	Ankazobe (18° 19' S, 47° 07' E)	biting humans indoors	April 1996
Tanzania	Bagamoyo (6° 26' S, 38° 55' E)	resting indoors	December 1992
Botswana	Shakawe (18° 10' S, 21° 43' E)	resting indoors	March 1994
South Africa	Komatipoort (25° 26' S, 31° 57' E)	biting humans outdoors, CO_2 -baited net traps	January 1996, 1997
	Thomo Camp, Giyani (23° 15' S, 30° 47' E)	pit collections	December 1997
	Mamfene (27° 23' S, 32° 16' E)	feeding on cattle	March 1998
Mozambique	Boane (26° 02' S, 32° 19' E)	resting indoors	May 1997
Côte d'Ivoire	Petionara (near Bouaké) (7° 42' N, 5° 0' W)	inside houses	June 1997
	Abidjan (15° 19' N, 4° 1' W)	inside houses	June 1997.
Senegal	Dielmo (13° 45' N, 16° 25' W)	indoors using pyrethrum spray catch	February 1998
	Kedougou (12° 33' N, 12° 11' W)	indoors using pyrethrum spray catch	October 1997
	Sankagne (13 ° 24' N, 13 ° 45' W)	indoors using pyrethrum spray catch	November 1997.

***A priori* identifications:**

All wild caught females were brought back to the Botha De Meillon insectary in the Department of Medical Entomology at the South African Institute for Medical Research (SAIMR). Female *An. funestus* group specimens were morphologically identified according to Gillies and De Meillon (1968) and Gillies and Coetzee (1987). Females were blood fed and put into separate tubes with damp filter paper to induce egg laying. The insectary conditions were constant at 24-25°C and 80% relative humidity. Day and night cycles were simulated with dawn and dusk periods of 45 minutes each. Egg batches were collected and morphologically identified. Female mosquitoes surviving after egg laying were transferred to fresh tubes and offered a blood meal. Thirty three hours after the females were fed, they were aneathetized using ether and the ovaries dissected into Carnoy's fixative. The rest of the body was stored either in 100% ethanol or desiccated on silica gel or in liquid nitrogen for further analysis.

Egg batches were transferred to small plastic bowls with or without aerated water. Larvae hatched after 2-3 days and those that did not, were incubated in a water bath at 37°C for 30-60 min to assist egg-hatching. A small number of hatched eggs per family were stranded on filter paper and stored in bottles containing cottonwool soaked with 80% ethanol. Larvae were fed on several different diets, from ground tropical fish food to ground desiccated yeast, ground desiccated yeast mixed with ground dog biscuits or combinations of these foods. Larvae were transferred to clean water as soon as it was noticed that an oily layer or bacterial growth had accumulated on the surface, as well as in certain circumstances where larvae started dying for no apparent reason. The fourth instar larval pelts were collected and preserved in 80% ethanol after pupation. The pupae were removed and transferred to a small dish inside a gauzed cage. Once adults emerged the pupal pelts were collected and stored in 80% ethanol together with the larval pelts.

Adult mosquitoes were kept alive in cages with a 10% sucrose solution. Where possible, ten adults (five males and five females) from each family were pinned and the remainder of the family were frozen in liquid nitrogen for use in DNA or protein studies. Four or more pelts per family were mounted and labelling data correlated with the pinned adults. Adults, eggs and larvae were identified using the keys of Gillies and De Meillon (1968) and Gillies and Coetzee (1987). Adults not pinned or frozen were pooled in one cage once they were identified, in order to attempt establishing a colony.

Chromosome identification

Half gravid ovaries were dissected from aneathetised female specimens by grasping the last abdominal segment with forceps. Ovaries were pulled out of abdomen while gently squeezing the abdomen between thumb and forefinger. Dissected ovaries were stored in Carnoy's fixative (3 parts ethanol mixed with 1 part of glacial acetic acid) at -20 °C. Chromosome preparations were done according to Hunt (1973) and Green and Hunt (1980). Chromosome identification was done according to Green and Hunt (1980) and Green (1982).

Mounting of fourth instar larval and pupal pelts:

Mounting of fourth instar larval and pupal pelts was performed as follows: The pelts were transferred from 80% to 100% ethanol for approximately 15 min to remove all traces of water. They were then transferred to xylene for 15-30 min. The pelts were mounted on slides in entellan (with xylene, alkylacrylate) (Merck, cat no. 7961) and covered with 20 x 20 mm cover slips. Slides and pinned adults from each family were labelled with species name, locality, method of collecting, collector, date and unique code identifying the family.

2.3 RESULTS AND DISCUSSION

2.3.1 *A priori* identification

2.3.1.1 Morphological identification

Table 2.2 summarizes the specimens collected with identification from various field collection trips. Morphological identification of wild caught female *An. funestus* group specimens was difficult when specimens were old or badly damaged. Egg morphology could be used to distinguish *An. rivulorum* and *An. leesoni* from *An. funestus* and *An. vaneedeni*. *Anopheles leesoni* was identified on egg morphology, with floats widely separated throughout their entire length with the frill occupying the full length of the egg and continuous between the floats (Gillies and De Meillon, 1968). The deck was uniformly narrow throughout the length of the egg. *Anopheles leesoni* larvae never hatched successfully from the eggs and no further morphological investigation could be done. The *An. leesoni* wild caught females were preserved on desiccant for DNA analysis.

Newly emerged *An. rivulorum* adults were identified by the orange-brown colour of the thoracic integument. The base of the club on the male palps was dark and morphologically similar to *An. funestus*. Larvae of *An. rivulorum* were characterized with the clypeus having the same pigmentation as in *An. funestus/An. vaneedeni*, but with the sutural setae branched from the base. The main abdominal plates were shallow with mainly 1 accessory plate observed in 90% of larval pelts studied (n=34). The remaining 10% of the specimens showed three accessory plates. Gillies and De Meillon (1968) indicated that the width of the deck between the floats of *An. rivulorum* is usually greater than that observed for *An. funestus*. Preserved eggs of families identified as *An. rivulorum* were removed from the alcohol bottles and airdried. Using a micrometre eye piece and 40 x magnification, the width of the decks were measured directly in front of the lobed tubercles and halfway between the lobed tubercles and the floats. Lobed tubercles are found at the anterior end of the egg on the dorsal surface (Harbach and Knight, 1980).

Table 2.2 Species identification after *a priori* identification on wild caught specimens.

Country	Locality	Identification	Families obtained
Madagascar	Ankazobe	*An. funestus*	26
Tanzania	Bagamoyo	*An. funestus*	23
Botswana	Shakawe	*An. funestus*	5
South Africa	Komatipoort	*An. vaneedeni*	26
		An. rivulorum	19
	Thomo Camp, Giyani	*An. vaneedeni*	208
		An. leesoni	No larvae from 2 females
		An. rivulorum	8
	Mamfene	*An. rivulorum*	1
		An. vaneedeni	6
Mozambique	Boane	*An. funestus*	35
Côte d'Ivoire	Petionara	*An. funestus*	17
	Abidjan	*An. funestus*	41
Senegal	Dielmo	*An. funestus*	6 individuals
	Kedougou	*An. funestus*	6 individuals
	Sankagne	*An. funestus*	6 individuals

Anopheles rivulorum (n= 5 -10 eggs, 11 families) eggs measured approximately 0.05mm (0.045mm - 0.055mm) directly in front of the lobed tubercles and 0.045mm (0.05mm - 0.04mm) halfway between the tubercles and the floats. *Anopheles vaneedeni* (n= 8 - 10 eggs, 6 families) eggs measured approximately 0.025mm (0.02 - 0.3mm) directly in front of the lobed tubercles while the area halfway between the tubercles and the floats measured approximately 0.0125mm (0.01 - 0.02mm).

No difference in egg morphology was observed between *An. funestus* and *An. vaneedeni.* Larval morphology conformed to the descriptions of Gillies and De Meillon (1968) and Gillies and Coetzee (1987). The clypeus had two transverse bands of dark pigmentation as described in Appendix I. Sutural setae were simple and abdominal plates were large and pigmented, covering most of abdominal segments 4-7.

Anopheles funestus adult morphology was as described in Gillies and De Meillon (1968). A pre-sector pale spot was present on the wings in 35% of specimens examined (n= 65, 19 families) with the majority of *An. funestus* having no pre-sector pale spot. Legs were dark with only the tibia having a small apical white spot. The tarsomeres very occasionally had a few pale scales on the joints which were especially visible in young specimens. Male palps were always dark at the base of the club.

Anopheles vaneedeni specimens were identified by obvious pale spots on the joints of the tarsomeres although they were not present in a small proportion (10%) of specimens examined (n= 24, 12 families). Male palps in some specimens differed from those of *An. funestus* by the presence of a few pale scales at the base of the club (25%, n= 24). The remaining specimens were dark as in *An. funestus.* A pre-sector pale spot was present on the wings in 76% of *An. vaneedeni* specimens examined (n=46). De Meillon *et al.* (1977) found that only 3% of *An.*

vaneedeni lacked the pre-sector pale spot in comparison with *An. funestus* where 79% lack the pre-sector pale spot.

2.3.1.2 Chromosome identifications

Polytene chromosome analysis of seven *An. funestus* specimens from Mozambique all had the standard arrangement (Green and Hunt, 1980). Two specimens were heterozygous for 3b/+. A further 42% of specimens (n=1) were either heterozygous for inversion 5a/+, 3ab/++ or 3b/+ 5a/+. Two individuals (28%) did not show any inversions. Polytene chromosome analyses from Senegal were published by Lochouarn *et al.* (1998) and the following is a short summary. *Anopheles funestus* specimens from Dielmo all showed the standard chromosomal arrangement for arm 2. Arm 3 showed heterozygous inversions for a/+, b/+, a/b and b/b. The majority of specimens had the standard arrangement for arm 3 (58.7%). Arm 5 was found to be heterozygous for a/+ and a/a. Only 27.7% of specimens from Dielmo had the standard arrangement for arm 5. The Kedougou population showed a higher number of polymorphism in arm 2 (See Lochouarn *et al.*, 1998 for detailed frequencies). Arm 3 of Kedougou populations were characterised by 93.9% of specimens having the inversion arrangement ab/ab and 6.1% having the arrangement ab/b. Arm 5 showed the same inversions as found in specimens from Dielmo.

Sankagne populations were characterized by inversion 2s/s in 93% of specimens and 2s/+ in 7% of specimens. Only inversion 3b/+ were found in 7.3% of specimens. The standard for arm 5 were found in 45% of specimens and inversion 5a/+ and 5a/a were found in 50% and 3.5% respectively.

2.3.2 Rearing of wild caught *An. funestus* group

Extensive information is available on the larval habitats of *An. funestus* (Gillies and De Meillon, 1968), but few studies have been done on other members of the group owing to the difficulties of identification. One *An. vaneedeni* larva was caught on a field trip during 1999 to Thomo in the Giyani district in the Northern Province. The larva was found in a large pool surrounded by vegetation. All larval collections were made in the shallow part of the pool and no other *An. funestus* group larvae were collected. The pool was shaded by a large tree during a part of the day.

Leeson (1937) recorded finding *An. funestus* larvae in breeding sites where two thirds of each site received less than 30% light. Larvae reared under laboratory conditions in the dark never reached pupation (Jepson *et al.*, 1947). Shade is suggested to play a more important role in the lowering of the water temperature than providing the larvae with less light (Jepson *et al.*, 1947). *Anopheles funestus* larvae were collected in water bodies with pH values ranging between 7.4 and 8.4 and were found with other species such as *An. gambiae* group and *An. coustani* (Jepson *et al.*, 1947).

Larvae from wild caught female *An. funestus* group were reared in distilled water (pH 6.4). Owing to the formation of scum and bacterial growth, the water was changed one to three times per week. During 1999 fourth instar larvae from an *An. quadriannulatus* strain were put into bowls containing newly hatched *An. funestus* group larvae in order to help clear the water surface. This helped in preventing bacterial growth on the surface making it possible to feed the larvae more often. Larval growth was enhanced which increased the survival rate of the larvae. *Anopheles quadriannulatus* larvae were removed once they had pupated. When *An. funestus* group larvae reached second or third instar it was not necessary to put additional *An. quadriannulatus* larvae into the bowls.

The aerating of water did not increase the survival rate of the larvae and the food combinations tried were all equally successful. Jepson *et al.* (1947) concluded that the main factors controlling *An. funestus* breeding under natural conditions were temperature and food supply. They reported that the total life cycle of *An. funestus* varied between 21-30 days at 24°C. Time required for development from egg to adult *An. funestus* group reared in the Botha De Meillon insectary varied between 30-42 days.

2.3.3 Colonizing *An. funestus*

Service and Oguamah (1958) reported difficulty in colonization of *An. funestus* but they finally succeeded after three years. Egg production was of short duration and in small numbers, and the colony eventually died out. This colony was fed on human and guinea pig blood. Two years later a colony of *An. funestus* was successfully maintained for at least eight generations and it is unknown how long the colony survived subsequent to them publishing their results. The second colony was maintained on guinea pig blood alone. Larvae were reared in tap water and were fed on pure yeast. Adults of the second colony were kept in total darkness in a cage measuring approximately 30cm x 30cm.

Attempts to colonise *An. funestus* and *An. vaneedeni* were unsuccessful in this study. Since the mating behaviour of the *An. funestus* group is undescribed it is difficult to know what they require in their environment to stimulate mating. Female *An. funestus* kept in a 5L (21cm high) container were dissected after 14 days and it was found that they had not mated. These adults were transferred to a larger cage (1m) and the temperature was increased to 26°C. Mating took place and eggs were laid, but the adults died after 35-45 days and not enough eggs were laid to maintain the colony. *Anopheles vaneedeni* kept under similar conditions at 25°C never laid any eggs over a period of one month. Keeping the colony in the dark did not increase egg production.

Chapter 3

SEQUENCING

3.1 INTRODUCTION

Two methods exist for sequencing DNA fragments. The first method, Maxam and Gilbert, is used to a lesser extent than the Sanger method. Both methods generate a ladder of fragments of different sizes, which are labelled with either radioactivity or silver (Adams *et al.*, 1992). The different fragments are separated by using a high resolution polyacrylamide gel. Electrophoresis on a polyacrylamide matrix can separate fragments differing in as few as one DNA base. The Maxam and Gilbert method is the chemical fragmentation of either double or single stranded DNA by base-specific reactions (Adams *et al.*, 1992; Sambrook *et al.*, 1989). The Sanger dideoxy method is the primed synthesis of partial copies of the DNA to be sequenced, with random base-specific pre-mature-termination during chain elongation. Producing a ladder with different length fragments terminating in either of the four bases (A,C,G, or T) (Adams *et al.*, 1992; Old and Primrose, 1993 ; Sambrook *et al.*, 1989). The Sanger method of sequencing is most commonly used and can be done either manually or by automated sequencing.

Template DNA for sequencing can be obtained by cloning DNA to be sequenced into a plasmid vector or by using amplified PCR product as template DNA. I used sequencing in this study for the detection of species-specific DNA variation within regions of the ribosomal DNA (rDNA) and the mitochondrial DNA (mtDNA).

3.1.1 rDNA

rDNA plays an essential role in mRNA translation. These rDNA genes are transcribed, processed and assembled in the nucleolus to form the ribosome (Freifelder, 1986; Adams *et al.*, 1992). Protein biosynthesis takes place within the ribosome and involves the successive reading of codons on the mRNA by the aminoacyl-tRNA and the linking of the amino acids to form a polypeptide chain (Adams *et al.*, 1992; Freifelder, 1986).

A typical eukaryotic ribosome has an *S* value (S=Svedberg sedimentation value) of about 80 (80S) and consist of two subunits, a 40S and a 60S subunit. However, the sizes of subunits may vary over a 20% range from one organism to the next. Rat ribosomes are the best studied and the 40S subunit contains one 18S rRNA molecule and approximately 30 other proteins. The 60S subunit contains one 5S, one 5.8S and one 28S rRNA molecule and about 50 other proteins. The other proteins found are difficult to isolate and the exact amount involved and whether they are ribosomal or non- ribosomal proteins is unknown (Adams *et al.*, 1992).

Ribosomal RNA in eukaryotes represents more than 80% of the cellular RNA. To produce these amounts of RNA, the RNA is encoded by multiple genes (rDNA). The rDNA genes are transcribed by a rapid rate of one RNA polymerase molecule at 100bp/s, each transcribing at 30bp per second (Adams *et al.*, 1992). The speed of transcription is another way to ensure enough rRNA is produced to satisfy the demand during translation. Clusters of rDNA transcription units occur at the nucleolar organizers and each unit contains three of the four ribosomal RNA species, the 18S, 28S and the 5.8S (Adams *et al.*, 1992). Considerable variation occurs in the number, arrangement, size and chromosomal location of these rDNA units depending on the genus and taxa. The rDNA transcription units can occur at several different loci, as is found in *Drosophila melanogaster* where the loci for the rDNA are found on both the x and y chromosome (Adams *et al.*, 1992).

The fourth rRNA species in eukaryotes, the 5S gene, is not normally linked to the other ribosomal RNA species, but is dispersed through the genome in tandem arrays. In *Drosophila* the 5S gene appears on chromosome two, but in species such as *Xenopus* the gene occurs at the telomeres of most of the chromosomes (Adams *et al.*, 1992).

Apart from the ribosomal genes, the rDNA transcription unit in eukaryotes also contains the non-transcribed regions (Figure 3.1). The external transcribed spacer (ETS) contains the promoter and is generally downstream of the intergenic spacer region (IGS), which is highly variable in length (Collins *et al.*, 1990). The IGS region, also called the non-transcribed spacer (NTS) region, separates the transcription units from one another (Paskewitz and Collins, 1997). Between the 18S and 5.8S genes, an internal transcribed spacer region (ITS1) is found. The 5.8S and the 28S coding region are also separated by another internally transcribed spacer region called the ITS2.

Certain characteristics of the non-transcribed regions make the ITS1 and ITS2 possible candidates to examine the differences existing between species. These differences can be used to develop species-specific primers or probes to identify species. The first advantage of these regions is that they are not transcribed by RNA polymerase. Changes in DNA sequences are more harmful for some transcription products than for others and individuals who carry these harmful mutations are eliminated from populations by natural selection (Alberts *et al.*, 1994). Sequences that are not transcribed or translated to protein sequences are therefore under less evolutionary pressure and are therefore more free to diverge (Paskewitz and Collins, 1997).

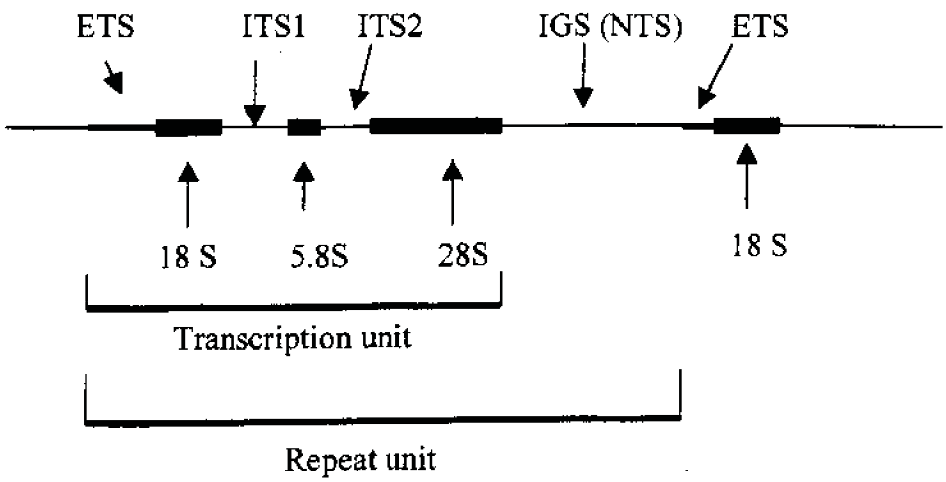

Figure 3.1 Schematic representation of a rDNA transcription unit (Adapted from Adams *et al.*, 1992)

Comparisons of substitution rates among different DNA regions in mammals also suggest that sequences with little or no functional constraints, accumulate mutations at a higher rate than fourfold degenerate coding sites (Porter and Collins, 1991). Sharp and Li (1987) estimated that at these non-coding sites, mutations occur at a rate of 16×10^{-9} mutations per site per year (*Drosophila* species). ITS (ITS1 and ITS2) sequences may have some overall length and nucleotide composition constraints, but they are probably free to accumulate a limited number of mutations at rates at least as high as those recorded for non-coding sites reported by Sharp and Li (Porter and Collins, 1991).

The second advantage of analysing the ITS1 and ITS2 regions are the relatively small size of