A SURVEY OF THE ANOPHELINE MOSQUITO FAUNA OF BOTSWANA, WITH SPECIAL REFERENCE TO THE MALARIA VECTORS

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Johannesburg, 1998

DECLARATION BY CANDIDATE

I declare that this thesis is my own work and that it has not been submitted for any degree to any other University.

R. Abdulla - Iman.

24th Day of January, 1998

ABSTRACT

This study was initiated in order to determine the identities and distribution patterns of the anopheline fauna, more especially the malaria vectors, in regions of Botswana prone to malaria epidemics. Field samples collected from Shakawe, Maun and Kasane over three consecutive years were subjected to morphological, cytogenetic, isoenzyme and PCR analyses. The results established that *Anopheles arabiensis* and *Anopheles funestus* were the predominant vector species.

Morphological and cytogenetic analysis of a salt-water breeder, similar to *An. azevedoi* and *An. listeri*, from Kasane, revealed the presence of a new species. This has been named in honour of Sir Seretse Khama, the late president of Botswana (1966-1980) and the manuscript has been submitted for publication. A description of the new species is provided here as "*An. listeri* sp.B".

Chromosomal analysis of the *An. gambiae* complex specimens established the presence of *An. arabiensis* only in all four localities over the study period. Arm 2R had one floating inversion, 2Rb, which is common in southern African populations of *An. arabiensis*. A Hardy-Weinberg analysis of each of the four populations, using one degree of freedom, revealed no significant deviations i.e. the observed frequencies of 2Rb+, 2Rb/b+ and 2Rb did not differ significantly from the expected frequencies. Arm 2L was uniformly monomorphic for the 2La arrangement, which is standard for *An. arabiensis*. Arm 3R was polymorphic for inversion 3Ra with the following frequencies: $3Ra^{+}$ (n=206), $3Ra/a^{+}$ (n=46), 3Ra (n=0). The absence of the 3Ra homozygote suggests that it may be at a selective disadvantage in this environment, or, that it may be associated with a lethal gene.

Statistical analysis of the genetic variability at six isoenzyme loci in all four Botswana populations revealed a deficiency of heterozygotes (at the 0.01 significance level) in each of the populations sampled. These results suggest that the Botswana population is subdivided. The effects of population subdivision

(measured by the fixation index F_{ST}) together with the derived estimates of migration (Nm) revealed that: for the EST, GCD and AAT loci there is scope for genetic divergence resulting from random genetic drift; for the GDH locus, migration is a potent force acting against genetic divergence resulting from random genetic drift among subpopulations; for the XDH locus, the Nm values and F_{ST} are somewhat contradictory.

PCR analysis of the Botswana sample revealed the presence of *An. arabiensis* only.

The results of our four-year study suggest that *An. arabiensis* and, to a lesser extent, *An. funestus* are the vector species in Botswana.

DEDICATION

This thesis is dedicated to my husband, Mohamed Allie Khan and my mother, Zoobeda Begum Mayet.

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INTRODUCTION

1.1 MALARIA IN BOTSWANA: A HISTORICAL REVIEW

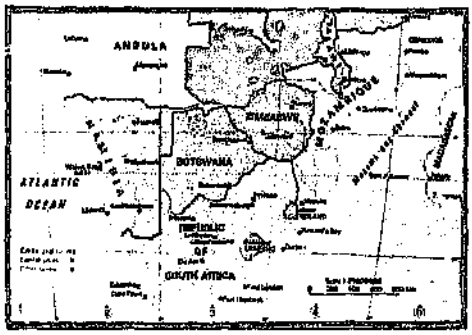
In Botswana, the transmission of malaria is unstable with seasonal outbreaks. One of the most severe outbreaks on record occurred in 1996, when more than 70 000 cases and 214 deaths were documented for the country. The intensity of these outbreaks is closely related to the rainfall pattern in each region. Approximately 50% of the country's population are exposed to malaria mainly between October and May i.e. the rainy season.

The geographical distribution of this disease in Botswana is not uniform, occurring within three main epidemiological belts namely the **northern, central** and **southern** belts.

The **northern belt** includes areas with relatively higher rainfall, namely the Northwest Sub-Districts (Ngamiland, Chobe) and parts of the Central District (Tutume) (Fig. 1). The transmission of malaria in these areas is relatively stable, accounting for more than 80% of the reported annual cases for the country. For example, during the malaria outbreak in 1996 the average infection rate in this particular zone was 20-40% whereas the national average was 3% (Ministry of Health, Botswana 1996). However, the mortality due to malaria in this region is generally lower in comparison to other parts of the country. This could be due to

FIGURE 1

Map of southern Africa (insert) and map of Botswana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BOTSWANA
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OCEAN
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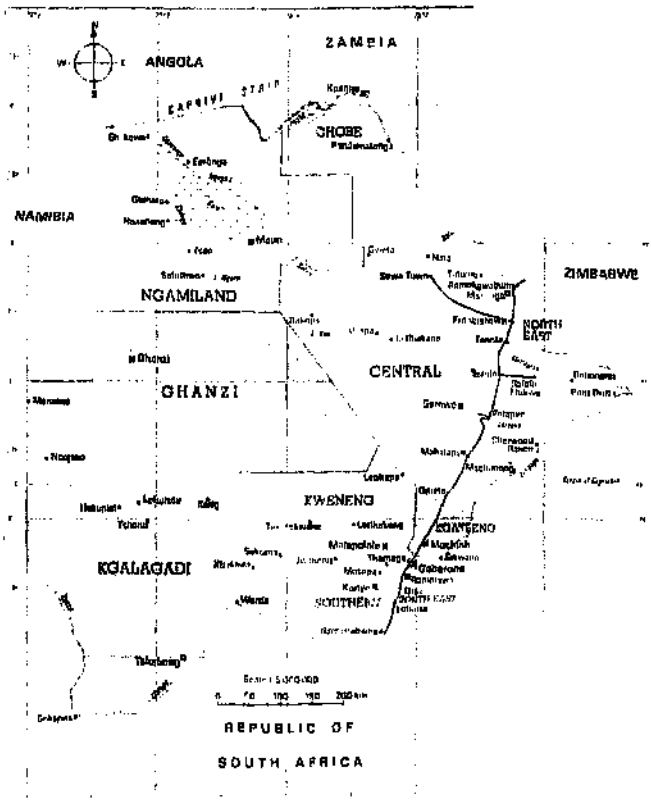
SOUTH AFRICA


ZAMBIA
ANGOLA
CAPRIVI STRIP
CHOBE
NAMIBIA
ZIMBABWE
NGAMILAND
NORTH EAST
CENTRAL
GHANZI
KWENENG
KGATLENG
KGALAGADI
SOUTHERN
REPUBLIC OF
SOUTH AFRICA

either the general awareness of the public health personnel and the community at large or, some immunity against the disease in the community, or, both. This zone borders Namibia and is separated from Zambia by the Caprivi Strip (Fig. 1). Control measures in place include (i) early case detection and treatment with appropriate drugs; (ii) vector control through indoor DDT spraying and (iii) personal protection by the use of repellents and insecticide impregnated bed-nets. Health education and advice on chemoprophylaxis for visitors is also provided.

The **central belt** includes the remainder of the Central District and the adjacent Ghanzi District (Fig. 1). Here the transmission is occasional, but somewhat higher in years when the rainfall is high. When epidemics occur they are accompanied by generally higher mortality rates. For example during the 1996 outbreak of malaria in this region the average case fatality ranged from 0.8%-1.2% in comparison to the national average of 0.2% (Ministry of Health, Botswana, 1996). The control measures in this belt focus mainly on disease surveillance and treatment with appropriate drugs. Routine vector control or chemoprophylaxis is not practised.

The **southern belt** consists of areas south of the Central District and parts of the North East District south of Francistown (Fig. 1). Transmission in these areas is sporadic. Focal episodes of transmission have been reported in years of heavy rainfall. No control measures are in place.

The magnitude of the malaria problem in Botswana

The annual incidence of malaria varies considerably ranging from 0-90 cases reported per 1000 of the population. Since the disease is seasonal no significant protection due to immunity is conferred on the population. This has been the key determinant of the clinical outcome of malaria in Botswana. Severe and complicated cases of malaria have been reported particularly in the northern belt and all age groups seem to be affected more or less equally according to the epidemiological reports generated by the Ministry of Health. The case fatality has remained significantly low ranging from 0-0.2%.

Studies on the anophelines

The anopheline fauna of Botswana (then Bechuanaland) listed in Gillies and De Meillon (1968) was compiled using published records and material seen in collections. It lists twenty-one species including *Anopheles funestus* Giles and *Anopheles gambiae* Giles as vectors of malaria (De Meillon and Leeson 1940). Since then, there have been a limited number of journal publications of more recent work carried out in Botswana (Mahon *et al.*, 76).

1.2 THE *ANOPHELES GAMBIAE* COMPLEX

The *Anopheles gambiae* complex of mosquitoes comprises six morphologically indistinguishable species varying in their vectorial capacity, behaviour and biology (Service 1985, Gillies and Coetzee 1987, Coluzzi 1992). Once regarded as a single, freshwater breeding species with a salt-water variant

(Evans 1938), the complex is now known to include three freshwater (*An. arabiensis* Patton, *An. quadriannulatus* (Theobald) and, *An. gambiae* Giles) two salt-water (*An. melas* Theobald, *An. merus* Donitz) and one mineral water (*An. bwambae* White) species.

A historical review: The earlier workers noted that populations of *An. gambiae* seemed to vary in their breeding places, resting sites and host preferences. Variation in breeding sites ranged from temporary freshwater pools to marshes and saline habitats (Evans 1938, De Meillon 1947). Adult females were seen to differ in their behaviour with some resting indoors and others outdoors after feeding (De Meillon 1947). There were also observable differences in host preference with some females feeding on (i) humans only; (ii) animals only (mainly cattle) and (iii) both humans and animals (De Meillon 1947).

In 1944, Ribbands (1944a, 1944b) presented both morphological and physiological evidence for the distinctness of *An. melas*, a salt-water adapted species found in West Africa. Muirhead-Thomson (1945) noted morphological differences between the eggs of *An. melas* and *An. gambiae* and went on to demonstrate reproductive isolation between the two due to the production of sterile hybrid male progeny. From this evidence, he concluded that *An. melas* was a distinct species (Muirhead-Thomson 1947). Bruce-Chwatt (1950) unfortunately, discounted the cross-mating experiments done by Muirhead-Thomson (1947) and his findings were, as a result, erroneously ignored. *Anopheles melas* was sunk into synonymy then called a "variety" and only recognised as a good species in 1964. Paterson (1962) carried out cross mating

experiments between East African salt-water breeders and freshwater *An. gambiae* and concluded that the salt-water form was a separate species. Similar hybridisation tests by Davidson (1962) and Davidson and Jackson (1962) helped differentiate additional, reproductively incompatible freshwater species formerly classified as "*An. gambiae*". Based on this evidence, Paterson (1963) concluded that these authors were dealing with two sibling species, to which Paterson *et al.* (1963) added a third from southern Africa. These were designated as species A, B and C (Paterson 1964). Davidson and Hunt (1973) then presented evidence for a sixth species, species D from hot mineral springs in Bwamba county, Uganda. In 1977, Mattingly assigned formal names to the three freshwater members of the *An. gambiae* complex i.e. *An. gambiae* (species A), *An. arabiensis* (species B), *An. quadriannulatus* (species C) and the two salt-water species i.e. *An. merus* in East Africa and *An. melas* in West Africa. In 1985, the mineralised water form, species D, was named *An. bwambae* (White 1985).

Biology: The two most efficient vectors are *An. gambiae* and *An. arabiensis*. *Anopheles gambiae* is endophilic and anthropophilic and exhibits a tendency to remain indoors after feeding; making it susceptible to indoor spraying campaigns (Davidson and Draper 1953, Gillies 1954). *Anopheles arabiensis* is (i) endo-and exophilic and (ii) zoophilic and anthropophilic and is therefore less affected by indoor spraying campaigns (White 1974, Service *et al.*, 1978). *Anopheles quadriannulatus* is predominantly zoophilic and not a vector (Boreham 1975, White *et al.*, 1980). *Anopheles melas* is a vector of malaria in West Africa (Bryan 1979, 1983) and *An. merus* is a vector in East Africa (Bushrod 1981, Mosha and

Petrarca 1983) though not in Kwazulu Natal (Theron 1978). Both *An. melas* and *An. merus* show lower sporozoite rates than *An. arabiensis* and *An. gambiae* (White 1974, Bushrod 1981, Bryan 1983). *Anopheles bwambae* has a very limited distribution and is therefore only implicated in the local transmission of malaria (White 1973).

Distribution: *Anopheles gambiae* and *An. arabiensis* are sympatric over most of their range i.e. most of sub-Saharan Africa. *Anopheles quadriannulatus* occurs in the south eastern parts of Africa, Zanzibar and Ethiopia. *Anopheles melas* is restricted to coastal West Africa, while *An. merus* occurs on the eastern side of the continent, not restricted to coastal areas. *Anopheles bwambae* is found only in the hot mineral water springs of the Semliki Forest, Bwamba county, Uganda (Gillies and Coetzee 1987).

1.3 SPECIES IDENTIFICATION OF THE *AN. GAMBIAE* COMPLEX

Since the six species that comprise the *An. gambiae* complex vary in their vectorial capacity and behavioural traits with two to four species occurring in sympatry over their geographical distribution (Coetzee *et al.*, 1993b), it is imperative that they be correctly identified to assess their impact on the transmission of malaria parasites.

Various techniques have been used in attempts to identify the species with varying success. These are briefly described below.

1.3.1 Morphological analysis

Absolute morphological features for conclusively separating *An. gambiae*, *An. arabiensis* and *An. quadriannulatus* do not exist, despite intensive studies carried out on them in different laboratories (Coluzzi 1964, Green 1971, White and Muniss 1972, Reid 1973, Coetzee 1986, Coetzee 1989). Variation observed in the means and ranges of certain characters in every stage of the life cycle of the mosquito, afford little scope for identification purposes especially under field conditions. Multivariate analysis of multiple morphometric data appears to offer a method for discriminating between the freshwater species but collection of the necessary data is tedious and time consuming (Coetzee 1989, Petrarca *et al.*, 1994). Morphological studies have been carried out on all the life stages of these mosquitoes. Each will be discussed briefly.

Eggs: As early as 1945, Muirhead-Thomson was able to distinguish eggs of *An. melas* from those of *An. gambiae* by measurement of the deck width. In East Africa, Kuhlow (1962) established that the eggs of *An. merus* were slightly larger than those of the three freshwater species. These findings were later supported by Paterson (1963). *Anopheles melas* eggs were later found to be longer and broader than those of the three freshwater species (Coluzzi 1964). However, no differences between the eggs of the freshwater species seem to exist.

Larvae: *Anopheles melas* could be distinguished from the freshwater species in West Africa by the larval pecten (Ribbands 1944a, 1944b). The number of branches on the inner shoulder hair was useful in distinguishing between *An.*

gambiae and *An. arabiensis* only in areas where *An. quadriannulatus* was absent (Green 1971).

Pupae: No characters for distinguishing between the pupae are known. Coluzzi (1964) seemed to find pupal setae of some use in his investigations however Coetzee (1989) found little use for these characters in distinguishing between members of this complex in southern Africa.

Adults: *Palpal ratio:* The most reliable character for distinguishing specimens of *An. merus* and *An. melas* from freshwater *An. gambiae* and *An. arabiensis* is the ratio of the length of the 4th and 5th segments of the female palp to the 3rd segment (Coluzzi 1964); there is less than 10% overlap in this index. Bryan (1980) found that palpal ratios could be utilised to identify 96.2% of *An. melas* and 91.59% of *An. gambiae* when they occurred together in the absence of other members of the complex. Bushrod (1981) utilised palpal ratios to distinguish *An. merus* from *An. gambiae* in Tanzania with very slight overlap in the values for this index. Coetzee (1989) used a multivariate discriminant analysis, which showed that by plotting palpal ratio against sensilla number, a good discrimination for *An. merus* could be obtained. *Antennal sensillae*: Coluzzi (1964) used this character to separate *An. gambiae* from *An. merus*. Bushrod (1981) found a very slight overlap between *An. gambiae* and *An. merus* in Tanzania. *Hind leg banding patterns*: Coetzee *et al.* (1982) used these to separate field collected specimens of southern African populations of An. *arabiensis* and *An. gambiae* from *An. quadriannulatus* and *An. merus*. Even though no overlap was detected, Coetzee (1986) later stated that

these results might not be valid for all areas on the African continent. In fact, a study carried out by Sharp *et al.* (1989) in Kwazulu Natal showed that *An. quadriannulatus* and *An. merus* could be identified correctly using Coetzee *et al's.* (1982) measurements however only 56% of *An. arabiensis* could be correctly identified in this region.

1.3.2 Salinity tolerance tests

Ribbands (1944b) investigated the salt-water tolerance of a variety of *An. gambiae* larvae. The result was that he could distinguish freshwater *An. gambiae* from *An. melas* by placing individual egg batches in distilled water. On hatching out, the 1st stage larvae are transferred to a solution equivalent to 75% seawater (23.5g NaCl/litre). Survival for two hours is indicative of *An. melas.*

Muirhead-Thomson (1951) then went on to differentiate between freshwater *An. gambiae* from East Africa and *An. merus.* These tests require live first instar larvae and only those larvae preferring saline habitats (*An. merus*) will survive the experimental procedure.

1.3.3 Cross mating experiments

Muirhead-Thomson (1948) was the first to demonstrate reproductive incompatibility in crosses between *An. gambiae* and *An. melas* by the production of sterile male hybrid progeny. Davidson (1956) while working on the genetics of insecticide resistance, noticed that crosses between adults from the north with those from the south of Nigeria gave rise to male progeny with atrophied testes. Davidson and Jackson (1962) crossed different West African freshwater

populations of *An. gambiae* and came up with two groups, which they called A. and B. Burgess (1962) established male sterility when he crossed *An. melas* with freshwater *An. gambiae*. Paterson (1962) and Kuhlow (1962) crossed *An. merus* from East Africa with freshwater "*An. gambiae*" to produce sterile males. Paterson *et al.* (1963) crossed species A and B with an "unknown" which they then went on to name species C (*An. quadriannulatus*). Davidson and White (1972) and Davidson and Hunt (1973) did similar crossing experiments in order to establish the status of *An. bwambae* from Uganda.

Original crosses were between biologically different populations i.e. freshwater versus salt-water. Subsequently, this method was used to cross *unknown* specimens with *reference* strains. If the offspring are from conspecific crosses, fertile hybrid males are produced and if they are from interspecific crosses, the F_1 males are always sterile. Predominantly male offspring are produced when *An. gambiae* and *An. arabiensis* males are crossed with females from *An. quadriannulatus*, *An. melas* and *An. merus*. Davidson *et al.* (1967) give results for all possible crosses between the five species recognised at that time. Hybrids can be confirmed by the presence of (i) atrophied and non-functional testes and (ii) asynapsis of polytene chromosomes from the salivary glands of 4th instar larvae and ovarian nurse cells. However, the technique is time consuming and laborious and requires well-established colony material and expertise in the investigator. It is seldom used today for routine identification purposes.

1.3.4 Cytogenetic analysis

Characteristic banding patterns of the giant polytene chromosomes found in the ovarian nurse cells of half-gravid females and also in the salivary glands of 4th instar larvae were found to be highly accurate for species identification in the *An. gambiae* complex (Coluzzi and Sabatini 1967, 1968, 1969, Green 1972, Hunt 1972, Davidson and Hunt 1973, Hunt 1973, 1987). The distinct banding patterns observed on the X chromosome of *An. arabiensis* separates it from the other members of the *An. gambiae* complex. *Anopheles merus* and *An. gambiae* share the same X chromosome banding pattern, however, fixed inversion "op" on arm 2R of the autosomes separates one from the other. *Anopheles melas*, *An. quadriannulatus* and *An. bwambae* also share an X banding sequence but can be separated by various fixed differences on their autosomal chromosomes.

Some of the disadvantages of this technique are that only half-gravid females and 4th instar larvae can be used and a high level of expertise is required to interpret the banding patterns. The method has the advantage of being cheap, samples can be stored in Carnoy's fixative in the field for later identification, and it is highly accurate. While chromosomal rearrangements have provided a reliable diagnostic tool for the six named species, polymorphic inversion frequencies in *An. gambiae* and *An. arabiensis* populations in West Africa (Bryan *et al.*, 1982, Coluzzi *et al.*, 1985) indicate that positive assortative mating is taking place, i.e. there is more than one species within each of these taxa.

1.3.5 Isoenzyme electrophoresis

Electrophoretic techniques to separate isoenzymes followed by histochemical staining were first applied to the *An. gambiae* complex by Mahon *et al.* (1976). They were able to separate *An. gambiae*, *An. quadriannulatus*, *An. arabiensis* and *An. merus* on the distribution of allele frequencies of three isoenzyme loci. Miles (1978) separated all six members of the complex using species-specific isoenzyme patterns. He incorporated his findings into a biochemical key (Miles 1979). The diagnostic systems include EST 1, GOT, ODH and SOD. The technique has been used for identification purposes by many researchers (Marchand and Mnzava 1985, Coetzee and Hunt 1986, Hunt and Coetzee 1986, Mnzava *et al.*, 1989, Coetzee *et al.*, 1993a, Braack *et al.*, 1994).

The advantages of isoenzyme electrophoresis are: (i) it can be carried out on crude extracts i.e. purification of the enzymes is not required; (ii) it is simple to perform and interpret and (iii) large samples can be processed in a relatively short time. The disadvantages include having to either keep specimens alive or stored in liquid nitrogen in the field and the need for sophisticated and expensive laboratory equipment. The results of this procedure have to be interpreted with care as some overlap in diagnostic systems have been recorded (Mahon *et al.*, 1976, Miles 1979, Marchand and Mnzava 1985, Mnzava *et al.*, 1989, Coetzee *et al.*, 1993a). Since the gene frequencies of diagnostic allozymes may vary geographically, it has been recommended that researchers check their results against chromosomally identified specimens (Hunt and Coetzee 1986).

1.3.6 Cuticular hydrocarbon analysis

This method involves the extraction and the analysis of chemically stable cuticular components using liquid gas chromatography. Carlson and Service (1979, 1980), Hamilton and Service (1983) and Phillips *et al.* (1988) used this method in an attempt to identify *An. gambiae*, *An. arabiensis* and *An. melas*. The accuracy of this method is limited, as there seems to be some overlap between the species investigated. The method is also of little practical value in the field as it is time consuming and involves complicated and expensive gas chromatography equipment.

1.3.7 DNA – based methods of identification

DNA probes

DNA probe-based methods have been investigated with a view to using them extensively in the field identification of the members of the *An. gambiae* complex. DNA offers several advantages: (i) the molecular structure is stable in dessicated or alcohol stored specimens; (ii) most DNA differences are not specific to sex or developmental stage and (iii) if detection methods are based on differences in highly repeated components of the genome, the DNA probes can be very sensitive (Collins *et al*, 1988). One disadvantage is that the procedure is very lengthy. Collins *et al.*, (1987) produced a probe that could distinguish between *An. arabiensis*, *An. gambiae* and *An. melas*. Tests against chromosomally identified specimens proved 100% accurate (Collins *et al.*, 1988). Gale and Crampton (1987a) developed two DNA probes; one could separate males of *An. arabiensis* from those of An. *gambiae* and the other could separate

An. arabiensis and *An. gambiae* from the salt-water tolerant species. Initially these probes could distinguish *An. quadriannulatus* from all except *An. gambiae*. Gale and Crampton (1987b) later developed a probe that overcame this problem. Male specific probes like these remain unsatisfactory. Gale and Crampton (1988) squashed specimens onto a membrane to enable more specimens to be processed in a shorter time. Membranes could be stored indefinitely but the method was cumbersome, involved radio labelling, assumed conspecific mating and depended on the relative intensity of reactions for identifications. Hill *et al.* (1991) developed a non-radioactive method using three oligonucleotide probes and squash blots which could identify the five main *An. gambiae* complex members but could only differentiate between males of *An. arabiensis* and *An. gambiae*. Field tests in Tanzania and S ica (unpublished data) showed overlapping results which were unsatisfactory.

The Polymerase Chain Reaction (PCR) method

Paskewitz and Collins (1990) introduced an identification system based on amplifying species-specific DNA fragments using PCR. They produced three "primers" derived from rDNA sequences that separated *An. arabiensis* from *An. gambiae*. The PCR method utilises a universal plus stranded primer which is derived from a conserved region at the 3' end of the 28S rDNA coding region and two species-specific minus stranded primers derived from the intergenic spacers. The universal primer reacts differently with the species-specific primers: it produces a 1.3 kb DNA fragment when the DNA from *An. gambiae* is used as a

template and only a 0.5kb fragment when the DNA from *An. arabiensis* is used. These products can then be separated electrophoretically on an agarose gel.

Scott *et al.* (1993) extended this work to produce four species–specific primers that could distinguish between (i) An. *quadriannulatus* (ii) An. *arabiensis* (iii) An. *gambiae* and (iv) An. *merus* and *An. melas*. While the primers for the last two species produce identically sized DNA fragments, this does not pose any practical problems since the two species are allopatric in distribution. Townson and Onapa (1994) used rDNA-PCR to identify *An. bwambae*.

The PCR method has been tested: (i) against chromosomally and enzymatically identified field specimens (Paskewitz *et al.*, 1993) where only one identification out of 217 was disputed; (ii) on southern African material against enzymatically identified specimens (Bredenkamp and Sharp 1993) with no dispute and (iii) on Tanzanian material against 412 chromosomally identified specimens with only three misidentifications (Van Rensburg *et al.*, 1996).

The advantages of this method are that any life stage or sex of the mosquito can be used, dried or alcohol preserved field specimens can be utilised, very small portions (e.g. DNA from one leg) of the mosquito DNA can be amplified leaving the remaining parts for use in additional analyses and, finally, the technique is fairly simple and the results easy to interpret. Some shortcomings which may limit the extensive use of PCR include the need for expensive laboratory equipment and chemicals, the use of ethidium bromide (a mutagen, for visualising the species-specific DNA bands), and the need to maintain the sterility of the reagents and equipment.

1.4 THE *ANOPHELES FUNESTUS* GROUP

Anopheles funestus is arguably second only to *An. gambiae* as a vector of malaria, and, in some areas, may even be responsible for the major part of transmission. Studies by Evans and Leeson (1935, 1937) on larvae, first established the existence of distinct "varieties" of *An. funestus*, most of which have subsequently been recognised as good species. It has become customary to refer to the currently recognised members as being part of the *An. funestus* group. Except for *An. funestus* which is anthropophilic, studies have shown that other members of this group appear to be mainly zoophilic although they also readily bite man outdoors in the absence of other hosts (Gillies and De Meillon 1968).

Anopheles funestus is widespread and abundant over the whole sub-Saharan region, wherever there is enough permanent water and very little or no use of residual insecticides. The northern most records are from the Niger River and in the south it extends to northern parts of Namibia, Botswana and as far south as Maputo, Mozambique and, historically, Kwazulu-Natal in South Africa. It also occurs in the Comoros and Madagascar, but has been eradicated from Mauritius (Gillies and De Meillon 1968). Populations of *An. funestus* are relatively stable because they breed in fairly permanent waters and are known to play a big part in endemic malaria (Gillies and De Meillon 1968). *Anopheles funestus* also plays an important part in the transmission of bancroftian filariasis in rural areas (Taylor 1930, Gordon *et al.*, 1932, Henrard *et al.*, 1946, Kartman *et al.*, 1947, Smith 1955, Nelson *et al.*, 1962) and O'nyong-nyong fever in Uganda (Haddow *et al.*, 1960). *Anopheles aruni* Sobti is known from Zanzibar where females have been found to

bite man outdoors at night and both males and females can be caught in the daytime resting in shaded banks and tree bases (Gillies and De Meillon 1968).

Anopheles brucei Service is known only from Lokoja in northern Nigeria and nothing is known about the egg or adult biology. The larvae, however, have been found in shady forest streams and partially dried riverbeds (Gillies and De Meillon 1968).

Anopheles confusus Evans and Leeson seems to be confined to plateau areas of eastern Africa from Kenya and Ethiopia to South Africa. It is a widespread species in Zimbabwe. Not much is known about the adult biology and it is presumed to be a zoophilic and outdoor species though De Meillon (1941) occassionally found it indoors. The larvae seemed to prefer slowly flowing streams (Gillies and De Meillon 1968).

Anopheles fuscivenosus Leeson is known only from Zimbabwe. Not much is known about the adult biology except that most specimens were collected in outdoor resting sites. The early stages of this specimen are not known (Gillies and De Meillon 1968).

Anopheles leesoni Evans is a widespread but localised species in the savanna regions of eastern and west n Africa. Its presence has been recorded in Ethiopia, Kenya, Uganda, Central African Republic, Angola, Mozambique and South Africa. In West Africa, it is known from Mali, Ivory Coast and northern Nigeria. Although occasionally collected in houses (Evans 1931) it is usually collected in outdoor resting sites and presumed to be zoophilic (De Meillon 1933, 1936, Leeson 1937). The larvae are mostly found at the edges of slowly flowing streams (Gillies and De Meillon 1968).

Anopheles parensis Gillies is exophilic and zoophilic. It is common in the eastern African lowlands of Kenya, north-eastern Tanzania, Pemba Island, Swaziland and South Africa. It was first recognised in Tanzania after residual house spraying had eliminated local *An. funestus* populations. *Anopheles parensis* is not capable of maintaining the transmission of malaria (Gillies and Furlong 1964). The larvae are found in permanent swamps and ponds among reeds and vegetation, unlike other members of the *An. funestus* group which show a preference for streams and moving water (Gillies and De Meillon 1968).

Anopheles rivulorum Leeson is found mainly in eastern and southern Africa from Ethiopia through East Africa, Pemba, Zanzibar, Angola, Zimbabwe, Botswana, Mozambique and South Africa. It is also found locally in the West African savanna from Mali and the Ivory Coast to northern Nigeria. It is exophilic and mainly zoophilic, only occasionally being found in houses (Gillies and De Meillon 1968). It has been caught biting humans outdoors in fairly large numbers in Tanzania though no gland infections were found (Gillies and Smith 1960). Wilkes *et al.* (1996) contradicted these results by finding sporozoite-positive samples thus showing that *An. rivulorum* is, in fact, a vector in Tanzania. The larvae are usually found in gently flowing water along the sides of rivers and in irrigation channels in parts of East Africa (Gillies and De Meillon 1968).

Anopheles vaneedeni Gillies and Coetzee is essentially an outdoor biting species, frequently biting humans (De Meillon *et al.*, 1977, Smith *et al.*, 1977). In the daytime, females can be found resting in pit shelters. Laboratory tests showed that this species was fully susceptible to *P. falciparum* malaria though its involvement in the transmission of malaria is uncertain (De Meillon *et al.*, 1977).

The larval habitat of this species is similar to that of *An. funestus* (Gillies and De Meillon 1968).

1.5 SPECIES IDENTIFICATION OF THE *ANOPHELES FUNESTUS* GROUP

1.5.1 Morphological analysis

Identification of the *An. funestus* group is mainly based on egg and larval characters.

Eggs: The eggs of *An. funestus*, *An. aruni*, *An. parensis* and *An. vaneedeni* are indistinguishable. The eggs of *An. brucei* and *An. fuscivenosus* are unknown. *Anopheles confusus* differs from *An. funestus* in that the bosses on the chorion are smaller. *Anopheles leesoni* eggs are different from all others in the group in that the frill occupies the full length of the egg and is continuous between floats. *Anopheles rivulorum* eggs have much smaller bosses on the chorion than those of *An. funestus*, and the width between the floats is also usually greater (Gillies and De Meillon 1968).

Larvae: The larvae of *An. rivulorum* and *An. brucei* are indistinguishable from each other as are the larvae of *An. funestus*, *An. parensis*, *An. vaneedeni* and *An. aruni*. *Anopheles rivulorum/brucei* differ from the latter species by having greatly reduced abdominal plaques and branched head seta 8-C. *Anopheles confusus* and *An. leesoni* have abdominal plaques intermediate in size between *An. funestus et*

al., and *An. rivulorum/brucei* and differ from each other only by head seta 8-C which is branched in *An. leesoni* and simple in *An. confusus* (Gillies and De Meillon 1968).

Adults: There are some differences in the thoracic colouration, wing markings and size of pale areas of the male and female palps. These characters are, however, variable within species and none can be used for accurate identification (Gillies and De Meillon 1968).

1.5.2 Cytogenetic analysis

The polytene chromosomes from ovarian nurse cells of *An. funestus* were used as a standard and compared with those of *An. parensis* and *An. vaneedeni* (Green and Hunt 1980). All three species share the same X chromosome banding sequences. *Anopheles parensis* differs from *An. funestus* by two fixed inversions while *An. vaneedeni* is homosequential with the *An. funestus* arrangement, differing only in the presence of a polymorphic inversion on Arm 2. Crossing experiments between *An. vaneedeni* and *An. funestus* (Green and Hunt 1980) produced sterile hybrids. Green (1982) published a large amount of chromosomal data on the other members of the *An. funestus* group. A summary of his discussions on intraspecific inversion polymorphisms in the group is provided here. *An. funestus* from The Gambia provided additional important data to that from southern African populations (Green and Hunt 1980) in that the whole sample was monomorphic for the standard arrangement except that 3a occurred with a relative frequency of 0.06, this contrasted markedly with the highly

polymorphic populations of southern Africa (Green 1982); *An. rivulorum* inversions 4c and 5e were recorded for Zimbabwean samples but not found in populations from Kwazulu-Natal, relative frequencies of 0.83 for 4c and 0.94 for 5e were recorded (Green 1982); *An. leesoni* from Tete Pan, Kwazulu-Natal, showed heterozygotes for Xd in three out of the seven insects collected and samples from Sinoia, Zimbabwe, showed Xd but were not scored (Green 1982). This work by Green (1982) permits, for the first time, positive identification of wild-caught females. Previously, families had to be reared from wild-caught females for identification from the diagnostic characters in the immature stages.

1.6 OBJECTIVES OF THIS STUDY

The first objective of this study was to identify the occurrence of the anopheline fauna of northern Botswana in order to begin up-dating records that go as far back as 1940 (Gillies and De Meillon 1968). The second objective of this study included unequivocally identifying the malaria vectors in regions of northern Botswana prone to malaria epidemics by using a combination of the available identification methods (morphology, cytogenetics, isoenzymes and PCR) with a view to providing information to be utilised in the country's malaria control programme.

CHAPTER 2

MATERIALS AND METHODS

2.1 FIELD COLLECTIONS

Mosquitoes were collected either biting humans indoors or outdoors, resting indoors or feeding and resting in cattle enclosures. A list of sampled localities and numbers collected is presented in Table 1. Collection sites are mapped on Figs. 2 and 3. The larval collections were relatively fewer since they had to be transported over long distances from Botswana back to the Insectary at the South African Institute for Medical Research (S.A.I.M.R.) in Johannesburg.

All samples that were collected in Botswana were kept separately in tubes with damp filter paper at the bottom and transported either by road or by air to the Insectary in Johannesburg for rearing of F_1 progeny.

2 LABORATORY PROCEDURES

2.2.1 Rearing of live material

The Insectary is maintained at a constant humidity (85% RH) and a constant temperature (24°C). Illumination is effected by tungsten incandescent lights which are controlled by a time switch and dimmer system. This system provides a twelve-hour day with a transition period of 40 minutes between complete light and dark.

Egg batches obtained from wild caught *An. gambiae* females were carefully washed off the filter paper and placed in distilled water in individual plastic bowls. The emerging larvae were fed on a mixture of powdered dog biscuits and yeast until they reached late fourth stage development. At pupation and emergence of the adult, the discarded larval and pupal pelts were collected and later mounted in Entellan (Merck). Some adults were mounted and deposited in the museum housed at the S.A.I.M.R. This procedure ensured that immature pelts and adults were correlated for some specimens of the *An. gambiae* complex, all members of the *An. funestus* group and some specimens of *An. coustani* and *An. tchekedii*. The *An. listeri* collections from Kasane were treated in the same way except that each egg batch was split 50:50 between bowls containing (i) distilled water and (ii) 25% seawater (8g/l sea salt).

Remaining adult males of the F_1 progeny of the *An. gambiae* complex specimens were killed and their legs removed and stored in 70% isopropanol for PCR identification. The rest of the body was preserved in liquid nitrogen for isoenzyme electrophoretic identification. Remaining adult females of the F_1 progery of the *An. gambiae* complex also had their legs removed and stored for PCR identification their bodies frozen in liquid nitrogen for isoenzyme electrophoretic identification and, wherever possible, ovaries were cropped from half gravid females and stored in Carnoy's fixative for chromosomal identification. *Anopheles listeri* adult female progeny were offered blood meals in an attempt to obtain half-gravid ovaries for chromosomal analysis.

2.2.2 Chromosome preparations

Half gravid ovaries were removed from wild or F_1 progeny females of the *An. gambiae* complex and the *"An. listeri"* group. The terminal segments of the abdomen were grasped with fine forceps and the ovaries were pulled out of the body while gently squeezing the specimen between the thumb and forefinger. The ovaries were immediately placed in Carnoy's fixative (3 parts ethanol: 1 part glacial acetic acid) and left in a refrigerator (4°C) for a minimum of 48 hours before they were analysed. Chromosome preparations were made according to the method of Hunt (1973) and Green and Hunt (1980) as follows:

Ovaries were removed from the fixative and placed on a slide in a drop of 50% propionic acid. Using a stereo microscope (Mag. x 12) any extraneous tissue and remains of the blood meal were carefully removed. The ovaries were teased apart with the aid of two tungsten needles and then stained with a drop of lacto-acetic-orcein for 5-10 minutes (1g orcein in 25 ml glacial acetic acid plus 25 ml 85% lactic acid; this stock solution was diluted: 1 part stock solution to 3 parts 50% propionic acid). Two drops of 50% propionic acid were added to soften the tissue before a clean, siliconized coverslip was placed over it. The coverslip was tapped with a needle in order to break the cell membranes and spread the polytene chromosomes. The whole slide was then covered with filter paper, held firmly at either end, and the preparation rubbed with fingertip to remove any excess fluid and ensure that chromosomes were absolutely flat. The slide was then warmed briefly. Photographs were taken on 35mm film using a 100% APOPLAN oil

immersion objective. Kodak Technical Pan film 2415 was used for all the chromosome photographs, which were developed in Kodak HC110 at a dilution of 1:19 for 8 minutes at 20°C. Prints were made on Ilfospeed paper and developed in Kodak D163 for 2 minutes. All the chromosome preparations were examined and scored using a phase contrast Vickers photoplan microscope.

Identifications of the *An. gambiae* material were obtained by comparing them with the standard chromosomal map for *An. arabiensis* (Hunt 1988). The *An. listeri* chromosomes were compared with those prepared by Green and Hunt from Sautini, South Africa, published in Clements (1992, Fig. 1.2).

2.2.3 Isoenzyme electrophoresis

Anopheles gambiae group samples from Shakawe, (collected in 1994) were electrophoretically identified using a flat bed horizontal cooling system as outlined in Coetzee *et al.* (1993a). Subsequent samples were run on polyacrylamide gels using a modified version of the vertical gel electrophoretic technique outlined by L.E. Munstermann (pers. comm., unpublished laboratory notes). All gels were stained according to the methods of Mahon *et al.* (1976) and Miles (1978). See Appendix I for full details.

2.2.4 Polymerase chain reaction (PCR) analysis

In this procedure outlined by Van Rensburg *et al.* (1996), the use of non-extracted DNA from mosquito samples as a template provides a rapid and accurate method for routine identification of the *An. gambiae* complex. For most

of the specimens collected in Botswana, legs were removed, placed in sample tubes containing 70% isopropanol and refrigerated Preserved samples were later identified using the modified PCR method of Van Rensburg *et al.* (1996) which was adapted from the protocol of Scott *et al.* (1993). See Appendix II for full details.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 COLLECTIONS

Over three consecutive years (1993-1995), Botswana anophelines were collected either biting humans indoors or outdoors, resting indoors or feeding and resting in cattle enclosures. A number of localities were sampled (Figs. 2 and 3) and the numbers and species of anophelines collected is presented in Table 1.

3.2 RESULTS

Only one member of the *An. gambiae* complex, namely *An. arabiensis* was collected in each of the localities sampled over the study period. *Anopheles funestus* samples were collected in Mohembo village, Kauxwi hostel and Kasane village, however, none was found in Maun. *Anopheles coustani*, *An. pharoensis* and *An. tchekedii* were collected in each of the localities. *Anopheles listeri*, "*An. listeri* sp.B", *An. nili* and *An. rivulorum* were collected in the Kasane area. *Anopheles pretoriensis* and *An. rufipes* were found in Francistown and *An. squamosus* in Maun and Kasane (Table 1).

3.3 DISCUSSION

The survey carried out in Botswana over the study period (1993-1995) is by no means an exhaustive one. There are at least eleven additional anophelines cited for Botswana in Gillies and De Meillon (1968) which, as yet, remain to be

FIGURE 2

Map of northern Botswana showing collection areas (▲)

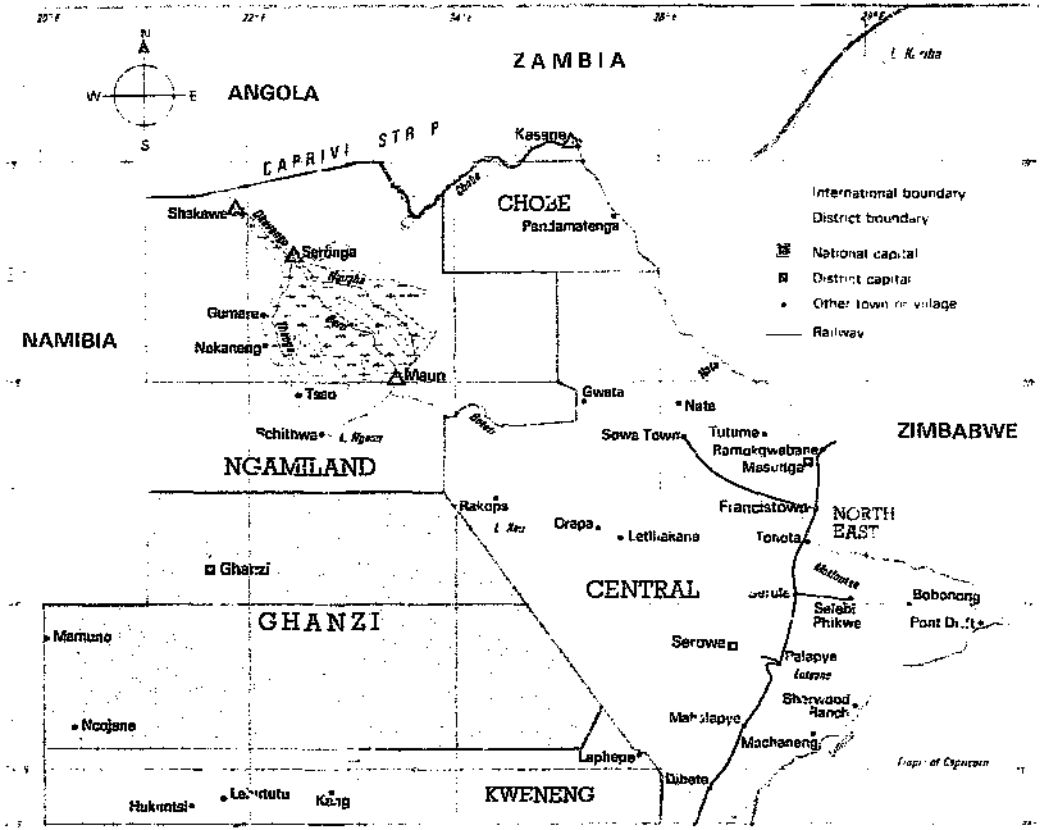

ZAMBIA
ANGOLA
NAMIBIA
ZIMBABWE
CAPRIVI STRIP
CHOBE
NGAMILAND
GHANZI
CENTRAL
NORTH EAST
KWENENG
Kasane
Pandamatenga
Shakawe
Seronga
Gumare
Nokaneng
Maun
Tsao
Schilthwa
Gweta
Nata
Sowa Town
Tutume
Ramokgwebana
Masunga
Francistown
Tonota
Orapa
Letlhakane
Rakops
Ghanzi
Mamuno
Ncojane
Hukuntsi
Kang
Lephepe
Dibete
Serowe
Palapye
Sherwood Ranch
Mahalapye
Machaneng
Selebi Phikwe
Bobonong
Pont Drift
International boundary
District boundary
National capital
District capital
Other town or village
Railway

TABLE 1. DETAILS OF COLLECTIONS OF ANOPHELINES IN BOTSWANA (1993-1995)

Species	Locality	Map Coordinates	Collection Methods	No. wild Adults(w)	No. wild Larvae	Total No. Adults(w+F_1)
arabiensis	Maun	20°01'S, 23°26'E	Resting indoors	11		27
			Cattle kraal	5		23
			Larval collections		9	9
				15	9	59
	Shakawe village('94+'95)	18°10'S, 21°43'E	Resting indoors	81+123		204
			Cattle kraal	0+2		2
	Mohembo village ('94+'95)	18°15'S,21°43'E	Resting indoors	33+12		45
			Cattle kraal	0+14		14
	Kauxwi hostel ('94+'95)	19°02'S, 22°08'E	Resting indoors	28+0		28
	Seronga village ('94+'95)	19°11'S, 22°42'E	Resting indoors	2+0		2
				144+151		295
	Kasane village	17°48'S 25°09'E	Outdoor manbiting	1		3
			Resting indoors	82		141
	Chobe Safari Lodge	17°48'S,25°06'E	Outdoor manbiting	8		21
	Mowana Lodge	17°48'S,25°11'E	Outdoor manbiting	6		13
	Kazangula road	17°48'S,25°19'E	Pig pen	4		11
			Cattle kraal	37		79
				138		268
coustani	Maun	20°01'S,23°26'E	Cattle kraal	7		7
	Shakawe village	18°10'S,21°43'E	Cattle kraal	3		3
	Mohembo pontoon	18°15'S,21°43'E	Outdoor manbiting	2		2
	Kasane village	17°48'S,25°09'E	Outdoor manbiting	4		4
			Cattle kraal	3		3
				19		19
funestus	Mohembo village	18°15'S,21°43'E	Resting indoors	19		76
	Kauxwi hostel	19°02'S,22°08'E	Resting indoors	1		5
	Kasane village	17°48'S,25°09'E	Resting indoors	10		47
				31		128
"listeri"	Kasane village	17°48'S,25°09'E	Resting indoors	6		20
	Kazangula road	17°48'S,25°19'E	Cattle kraal	45		66

			Pig pen	1		3
				52		89
nili	Kasane village	17°48'S,25°11'E	Resting indoors	8		8
	Mowana Lodge	17°48'S,25°11'E	Outdoor manbiting	5		5
	Lesoma village	18°02'S,25°20'E	Resting indoors	6		6
				19		19
pharoensis	Maun	20°01'S,23°26'E	Larval collections		5	5
			Resting indoors	4		4
			Cattle kraal	12		12
	Kazangula road	17°48'S,25°19'E	Cattle kraal	1		1
			Pig pen	1		1
	Shakawe village	18°10'S,21°43'E	Resting indoors	6		6
			Cattle kraal	11		11
				35	5	40
pretoriensis	Francistown	21°07'S,27°33'E	Larval collections		3	3
					3	3
rivulorum	Kasane village	17°48'S,25°09'E	Cattle kraal	15		15
				15		15
rufipes	Francistown	21°07'S,27°33'E	Larval collections		3	3
					3	3
squamosus	Maun	20°01'S,23°26'E	Larval collections		2	2
	Kazangula road	17°48'S,25°19'E	Cattle kraal	4		5
				4	2	7
tchekedii	Maun	20°01'S, 23°26'E	Cattle kraal	4		4
	Mohembo	18°15'S,21°43'E	Cattle kraal	3		3
	Shakawe	18°10'S,21°43'E	Cattle kraal	7		7
	Kazangula road	17°48'S,25°19'E	Cattle kraal	5		5
				19		19

identified in more current research. "*Anopheles listeri* sp.B" is a new species which should be added to the list whilst the "*An. gambiae*" of Gillies and De Meillon (1968) will need to be changed specifically to *An. arabiensis* in view of the results of this survey. More research is required at additional collection sites in Botswana before the anopheline fauna of the country can be realistically mapped and new distribution records generated.

CHAPTER 4

A DESCRIPTION OF *ANOPHELES (CELLIA) LISTERI* (DIPTERA, CULICIDAE), AND "*ANOPHELES (CELLIA) LISTERI* SP.B" (DIPTERA, CULICIDAE) FROM KASANE, BOTSWANA

4.1 INTRODUCTION

Anopheles listeri is a southern African species occurring in Zimbabwe, South Africa, Namibia and southern Angola (Gillies and De Meillon 1968). In Angola, the species shows high tolerance to salinity with the larvae being found in saline water that is greater than 1.5 times the concentration of sea water (Ribeiro and Ramos 1975). Gillies and De Meillon (1968), however state that the larvae are found in open sunlit pools and do not mention tolerance to salt water. Little is known about the adult biology of this species other than that in Namibia, the adults occur frequently in houses and bite humans readily (Gillies and De Meillon 1968). De Meillon (1931a) thought that *An. listeri* might be implicated in epidemic malaria transmissio in Namibia, despite the negative results of a very small number of dissections for malaria parasites. A morphologically similar species, *An. azevedoi* Ribeiro occurs frequently in highly saline pools along the south western coast of Africa, or in suitable areas inland in otherwise arid country. Larval habitats range from tidal pools, saline canals and saltpans to brackish wells

(Ribeiro 1974, Gillies and Coetzee 1987). The salinity of the most common breeding sites approached that of seawater (26-35g/l, NaCl) however, some larvae were found within extremely saline limits equivalent to 4-400% seawater. In conditions of low salinity, *An. azevedoi* occurred in association with *An. listeri* (Ribeiro 1974, Ribeiro and Ramos 1975). In the upper Karoo, Northern Cape Province the larvae of *An. azevedoi* were found in saline pools (14.1g/l, NaCl) along a river-bed in association with fairly large numbers of *An. listeri* (De Meillon and Van Eeden 1976). In Angola, adults of *An. azevedoi* were captured resting in houses, outdoors, particularly in crevices in the ground, in rocky cliffs and also in vegetation. Females were also collected biting humans outdoors (Ribeiro and Ramos 1975). Salivary gland dissections for sporozoites were carried out on 109 females with negative results (Ribeiro and Ramos 1975).

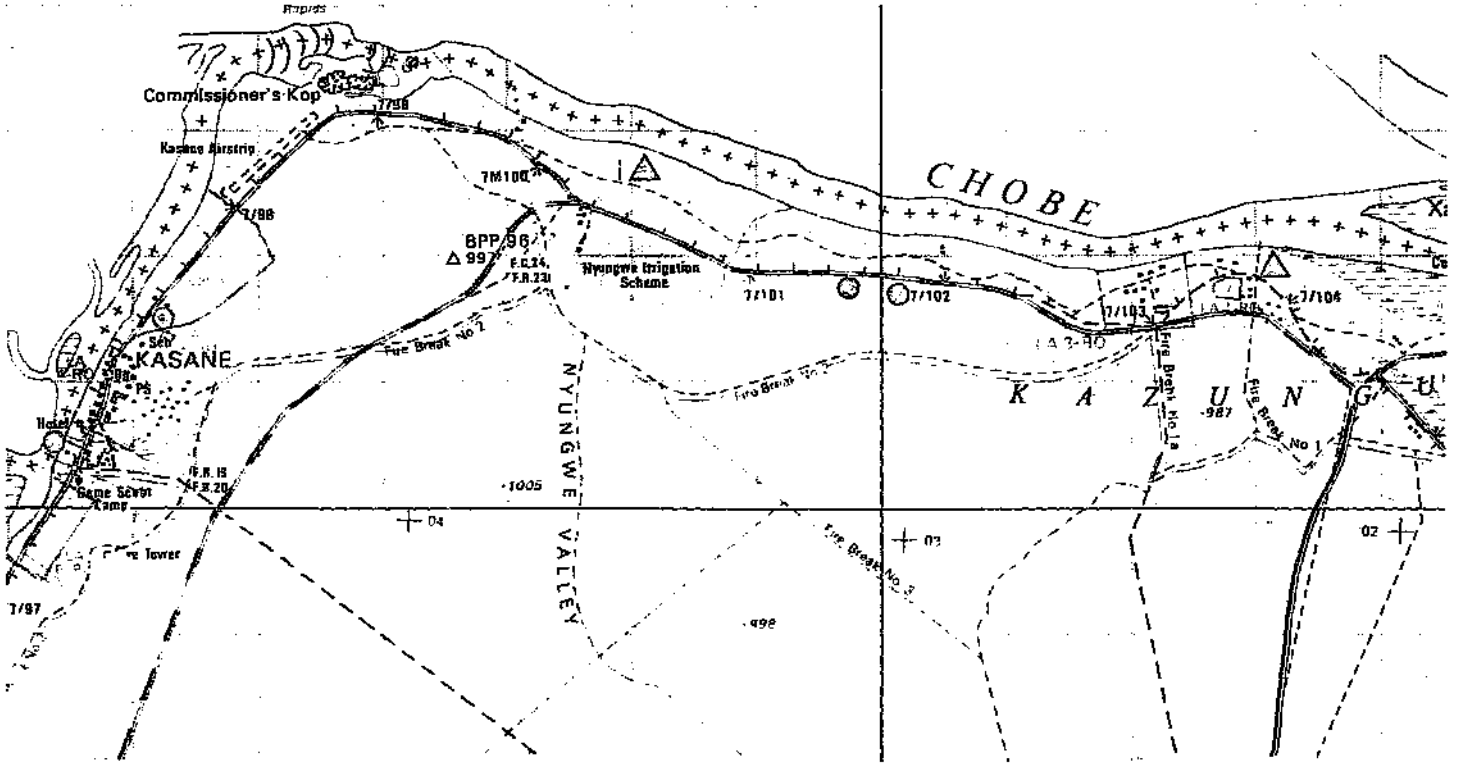
Collections of mosquitoes from Kasane in northern Botswana (Fig. 3), revealed two species, one indistinguishable from *An. listeri* and the other described here as new.

4.2 RESULTS

52 "*An. listeri*" females were collected, mainly from cattle enclosures (74%) resting indoors (23%) and in a pig pen (3%). These were transported to the S.A.I.M.R. Insectary. 20 laid eggs and those larvae reared in distilled water died during the first instar. Morphological examination of the egg batches revealed two distinct populations, one having floats (n=11) and the other without floats (n=9) (Figs. 5a, b). All egg batches produced larvae with well-developed

FIGURE 3

Map of Kasane, Chobe District, showing collection (◎) and location of saline hot springs (△)


Commissioner's Kop
Kasane Airstrip
7/98
7M100
BPP/96
Δ 997
F.R.24,
F.R.23
Nyangwe Irrigation Scheme
7/101
7/102
7/103
7/104
CHOBE
KASANE
Fire Break No 2
Fire Break No 1a
Fire Break No 1
NYUNGWE VALLEY
Game Scout Camp
Fire Tower
F.R.19
F.R.20
1005
04
03
02
7/97
498

abdominal palmate setae as in *An. listeri*, but unlike *An. azevedoi* which has the larval abdominal palmate setae reduced to simple hair-like processes (Ribeiro 1969, De Meillon and Van Eeden 1976).

Comparative studies were made between these two populations and the published descriptions of *An. listeri* as well as preserved museum specimens of *An. listeri*.

4.2.1 *Anopheles listeri*, Botswana

MATERIAL EXAMINED: *Anopheles listeri* (n=34 from 9 families) Botswana, Kasane, Chobe District. February 1995, Hunt and Khan coll., **cattle enclosure**: 17 females and 9 males; **indoors**: 3 females and 2 males; **pig pens**: 2 females and 1 male. **Museum material**: Opansi: 1 female and 5 males; Bethuli: 2 females; Lobito: 1 male; Fransfontein: 1 female; Eland mineral baths: 1 female and 1 male; Graaf Reinet: 1 female; Kuruman: 1 female.

BIONOMICS: Probably in saline hot springs where XRD analysis of soil samples indicated the presence of Halite (NaCl)(Appendix III, Table 5). In the laboratory, first instar larvae died in distilled water and survived in 25% seawater, indicating that *An. listeri* from Kasane is an obligatory salt-water breeder. Adult females were found indoors, biting humans (infrequently) and outdoors biting cattle (frequently) and pigs (infrequently).

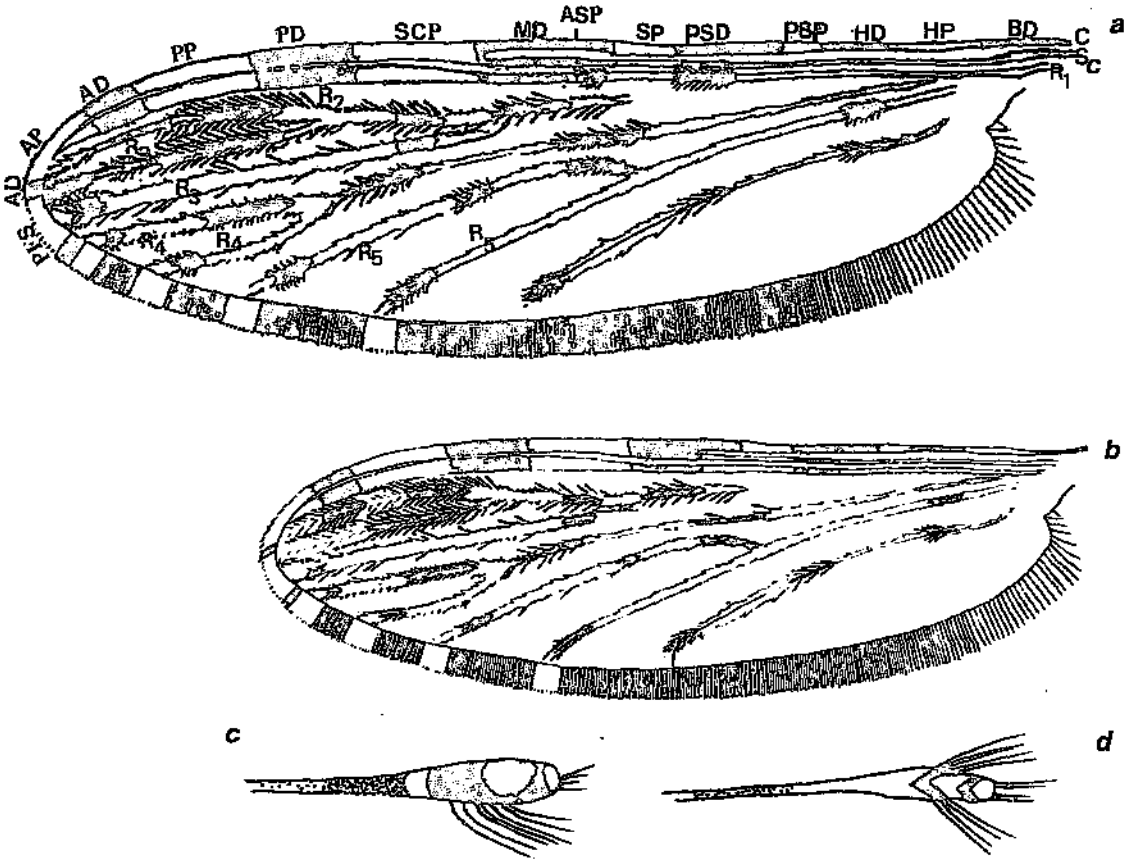
FIGURE 4

a: Female wing of *An. listeri* Botswana. C (costa); Sc (subcosta); PHP (prehumeral pale); BD (basal dark); PHD (prehumeral dark); HP (humeral pale); HD (humeral dark); PSP (presector pale); PSD (presector dark) SP (sector pale); MD (median dark); ScP (subcosta pale); PD (preapical dark); AD (apical dark); PFS (pale fringe spot); Veins (R_1, R_2, R_3, R_4, R_5).

b: Female wing of *An. listeri* from other localities (after Gillies and De Meillon 1968).

c: Male palp of typical *An. listeri* (after Gillies and De Meillon 1968)

d: Male palp of "*An. listeri* sp.B"


a
PP
PD
SCP
MD
ASP
SP
PSD
PBP
HD
HP
BD
C
C
C
AD
AP
AD
PVS
R1
R2
R3
R4
R5
R
b
c
d

MORPHOLOGICAL ANALYSIS:

Adult: Differs from *An. listeri* (in brackets) from other regions as follows: The range of wing length, 3.26 - 3.92 mm, makes it slightly smaller than *An. listeri* (3.4 - 5.0 mm) from the type locality of Msinga, Zululand (De Meillon 1931a). The wing field is largely pale, with the pale and dark areas well-contrasted (poor contrast between dark and pale areas). The presector dark areas of the costa and subcosta are approximately equal in length and partially fused (unequal and fused) while the apices of vein R_3 and vein R_4 have dark scales (pale scales) extending right up to the fringe spot (Fig. 4a). *Female Palps*: as in *An. listeri* (De Meillon 1931a). *Male Palps* (Figs. 4 c,d): two distinct male palps present with one resembling *An. listeri* (Fig. 4 c)(n=3) and the other different (Fig. 4d)(n=6) with a much paler overall appearance; the shaft is dark proximally followed by a long pale area; the club is widely pale at the extreme apex followed by a narrow, dark region preceding a wide pale area medianally; the pale base of the club merges with a long, pale area on the shaft. *Hind Leg*: the apex of the femur is dark whereas the base of the hind tibia is narrowly pale (Table 2) ranging in width from 0.0075-0.0125mm, (0.0075-0.01mm). The apices of the tarsomeres 1-4 are uniformly dark (pale or dark). *Male Genitalia*: as in *An. listeri*, De Meillon.

Pupa: Full setal counts (Belkin 1962) are given in Table 3. Differences between *An. listeri*, Botswana and *An. listeri* (in brackets) are as follows: Seta 1 on abdominal segment II with 7-9 branches (8-10); segment III with 6-8 branches (5-6); segment VII simple and same length as the segment (simple and longer than

TABLE 2

Differences between *An. listeri*, Botswana (n=34), "*An. listeri* sp.B" (n=55) and museum samples of *An. listeri* (n=30) and *An. azevedoi* (n=1).

Country	Location	Species	No. of specimens	Hind tibia base white (mm)	Hind tarsus	Wing length range (mm)	Apex vein 2.2 (R_2)	Apex vein 3 (R_3)	Dark areas B on costa and subcosta fused or separate?	Dark area B on subcosta (mean)(mm)
Angola	Lobito	*An. listeri*	2	0.01	dark	3.88-4.08	light	light	fused	0.3
South Africa	Upington N. Cape	*An. deaconi*	1	0.02	dark	3.68	light	light	separate	0.48
	Opansi, Kwazulu	*An. listeri*	11	0.0075-0.01	pale dark	3.72-4.08	light	light	separate	0.47
	Bethuli, Free State	*An. listeri*	6	0.01	pale	3.48-3.92	light	light	fused	0.28
	Eiland, Northern Province	*An. listeri*	4	0.01	dark	3.4-3.76	light	light	fused	0.4
	Graaf Reinet, E. Cape	*An. listeri*	2	0	dark	3.92-4.08	light	light	fused	0.43
	Kuruman, N. Cape	*An. listeri*	1	0	dark	4.72	light	light	fused	0.45
Botswana	Kasane, Chobe	*An. listeri*	34	0.0075-0.0125	dark	3.26-3.92	dark	dark	separate	0.68
	Kasane, Chobe	"*An. listeri* sp.B"	55	0.0075-0.0125	pale;dark	3.1[illegible]-3.60	dark	dark	separate	0.52
Namibia	Fransfontein	*An. listeri*	4	0.0075-0.01	pale	3.7-3.76	light	light	fused	0.32

TABLE 3

Full setal counts for pupal specimens (n=34) of *An. listeri*, Botswana

SETA	RANGE
MET	
10	2-4
11	3-4
12	2-4
ABD I	
1	>20
2	2-4
3	1
4	3-4
5	2
6	1-2
7	8-10
9	1-2
ABD II	
0	1
1	7-9
2	7-9
3	2-3
4	4-6
5	3
6	1
7	6-9
8	2-3
9	1
10	2-3
ABD III	
0	1
1	6-8
2	7-8
3	1-3
4	4-5
5	7-9
6	3-4
7	2
8	2-3
9	1
10	2-3

SETA	RANGE
ABD III	
11	1
14	1
ABD IV	
0	1
1	1-3
2	5-6
3	5-6
4	2-3
5	4-5
6	2-3
7	1-4
8	1-3
9	1
10	1
11	1
14	1
ABD V	
0	1
1	1
2	5-6
3	2-5
4	3
5	4-5
6	2
7	1-3
8	1-3
9	1
10	1
11	1
14	1-2
ABD VI	
0	1
	1
	5-6
3	2
4	1

SETA	RANGE
ABD VI	
5	4-5
6	3
7	1
8	2-3
9	1
10	1
11	1
14	1
ABD VII	
0	1
1	1
2	4-6
3	2
4	1
5	3-4
6	3-4
7	1
8	1
9	1
10	1-4
11	1-2
14	1
ABD VIII	
0	1
4	1
9	1
14	1
1	1
Paddle	
1	1
2	1

TABLE 4

Partial setal count of head and prothorax for larval specimens (n=29) of *An. listeri*, Botswana.

SETA	RANGE
HEAD	
2	1
3	1
4	1
5	4
6	3
7	3
8	3-7
9	4-8
10	3-7
11	9-15
12	2-4
13	3-5
ANTENNA	
2	1
3	1
4	5-8
PROTHORAX	
1	6-7
2	8-9
3	1

segment length). Seta 5 on abdominal segments III - VII with 7-9, 4-5, 4-5 and 4-5 and 3-4 branches (5-6, 5-6, 3-5, 3-5 and 1-3) respectively. Seta 9 on abdominal segments VI-VII simple and equal to or less than half the segment length (equal to or more than half length of segment).

Larva: Differences between *An. listeri* Botswana and *An. listeri* (in brackets) are as follows: *Antennal Seta*: seta 4 is fine with 5-8 (3-4) branches. *Shoulder Hairs*: inner and median hairs are branched and on small, separate basal tubercles; inner (1-P) 6-7 branches (4-5) and median (2-P) 8-9 branches (9-10). *Palmate Setae*: second abdominal rudimentary and leaflets are lanceolate and undifferentiated with 16(13) branches; third to seventh abdominal fully developed with 16(13) moderately broad leaflets, well defined shoulders and short, blunt-tipped filaments varying in length. A partial setal count for the head and prothorax on slides of larval specimens can be found in Table 4.

Egg: As in *An. listeri* except for the colour which is described as golden brown (Gillies and Coetzee 1987) instead of the shiny, silver-grey colour seen in the Botswana specimen. The boat-shaped egg has a broad upper surface (deck) which occupies the entire dorsal surface. There are darker, irregular patches across the middle and extreme ends of the deck where the bosses are relatively broader. The rest of the upper surface of the deck is lighter in comparison, consisting of finer, smaller bosses. The deck is surrounded by a well-developed frill and there is a complete absence of floats (Fig. 5b).

CYTOLOGICAL ANALYSIS: The chromosome banding patterns of *An. listeri*, Botswana (Fig.6) were found to resemble those of An. *listeri* collected in the Northern Province, South Africa (Fig. 7; from Clements 1992) with respect to arm X. However, the poor quality of the slide made it difficult to score the arms band for band with certainty (Fig. 6).

4.2.2 "*Anopheles listeri* sp.B" from Botswana

A description of this species has been submitted to the *Journal of the American Mosquito Control Association* for publication and copies of the two manuscripts are included as Appendix III here.

MATERIAL EXAMINED: "*Anopheles listeri* **sp.B"** (n=55 from 11 families) Botswana, Kasane, Chobe District. February 1995, Hunt and Khan coll., **cattle enclosure**: 28 females and 13 males; **indoors:** 9 females and 5 males.

DISTRIBUTION (Fig. 3): "*Anopheles listeri* sp.B" is known only from Kasane, Chobe District, Botswana.

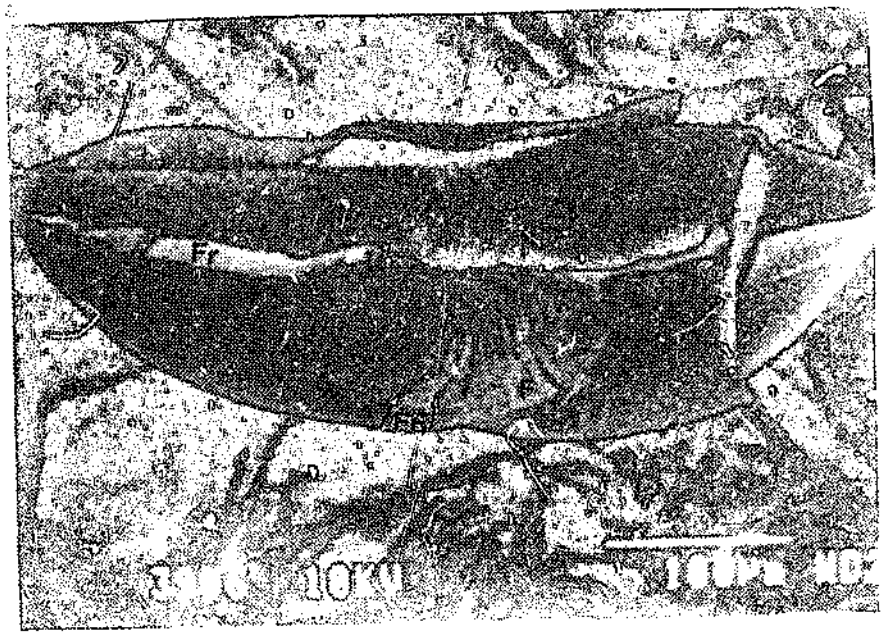
BIONOMICS: Unknown in nature, but probably in saline hot springs. An XRD analysis of soil samples taken from the hot springs near the type locality, showed the presence of Halite (NaCl)(Appendix III, Table 5). In the laboratory, first instar larvae died in distilled water and survived in 25% seawater, indicating that "*An.*

FIGURE 5

Scanning electron micrographs of:

a: "*Anopheles listeri* sp.B"; Eggs with floats (Mag. x 230) De (deck); Fr (frill); FR (float ridge); F (float). Dorso-lateral view.

b: *Anopheles listeri* (Botswana); Eggs without floats (Mag. x 230). De (deck); Fr (frill). Dorsal and dorso-lateral views.



b

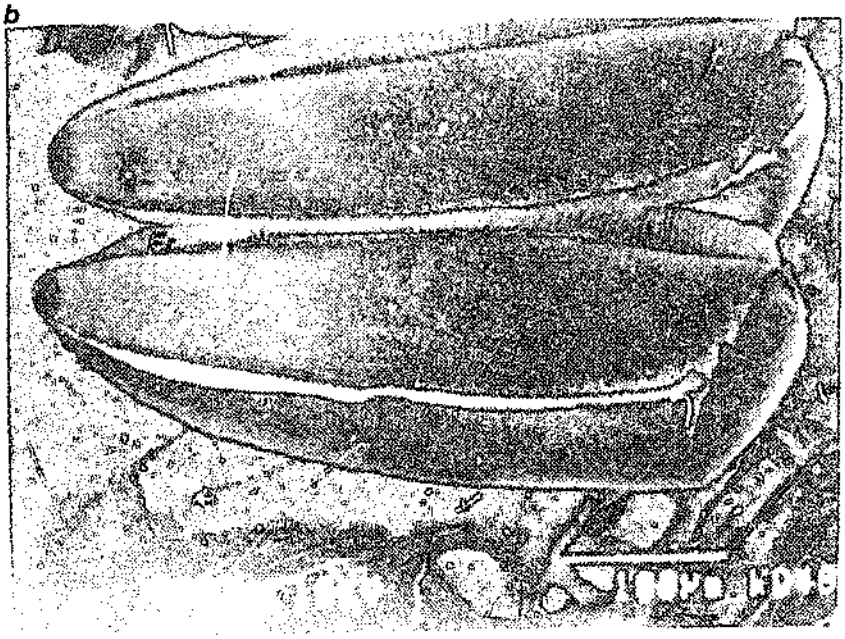


FIGURE 6

Polytene chromosomes from an ovarian nurse cell of *An. listeri* from Botswana showing Arms X, 2(2R), 3(2L), 4(3R), 5(3L).

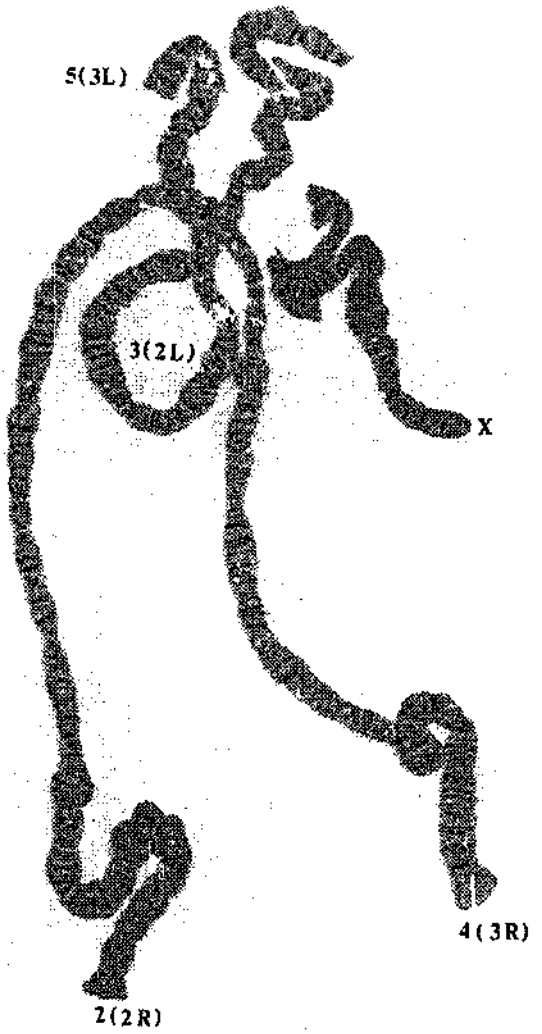

5(3L)
3(2L)
X
4(3R)
2(2R)

FIGURE 7

Polytene chromosomes from the ovarian nurse cells of *Anopheles listeri* (from Clements, 1992).

S(eL)
eg
2(eL)

listeri sp.B" is an obligatory salt-water breeder. Adult females were found indoors, biting humans (infrequently) and outdoors biting cattle (frequently).

MORPHOLOGICAL ANALYSIS:

Adult: Differs from *An. listeri* (in brackets) as follows: The range of wing length (3.16-3.60 mm) makes it much smaller than *An. listeri* (3.40-5.0mm) from the type locality Msinga, Zululand (De Meillon 1931a) and slightly smaller than *An. listeri* (3.26-3.92 mm) from Kasane. The wing field is largely pale (Fig. 8), with the pale and dark areas well-contrasted (poor contrast between dark and pale areas). The presector dark areas (PSD) of the costa and subcosta are approximately equal in length and separate (unequal and fused). The mean width of the dark area on the subcosta differs in the specimens collected and the mounted museum samples: *An. listeri* (0.38 mm); *An. azevedoi* (0.48mm); *An. listeri,* Botswana (0.68mm) and "*An.listeri* sp.B" (0.52 mm)(Table 2). The apices of vein R_3 and R_4 have dark scales (pale scales) extending right up to the fringe spot. *Female Palps*: as in *An. listeri* (De Meillon 1931a). *Male Palps* (Figs. 4 c,d): two distinct male palps present with one resembling *An. listeri* (n=7) and the other different (Fig. 5d) with a much paler

overall appearance (n=11). *Hind Leg*: the apex of the femur is dark whereas the base of the hind tibia is narrowly pale (Table 2) ranging in width from 0.0075-0.0125 mm (0.0075-0.01 mm). The apices of the tarsomeres 1-4 are uniformly dark (pale or dark). *Male Genitalia*: as in *An. listeri*, De Meillon.

FIGURE 8

Wing of *"An. listeri sp.B"* C (costa); Sc (subcosta); PHP (prehumeral pale); BD (basal dark); PHD (prehumeral dark); HP (humeral pale); HD (humeral dark); PSP (presector pale); PSD (presector dark); SP (sector pale); MD (median dark); ScP (subcosta pale); PD (preapical dark); AD (apical dark); PFS (pale fringe spot); Veins(R_1,R_2,R_3,R_4,R_5).

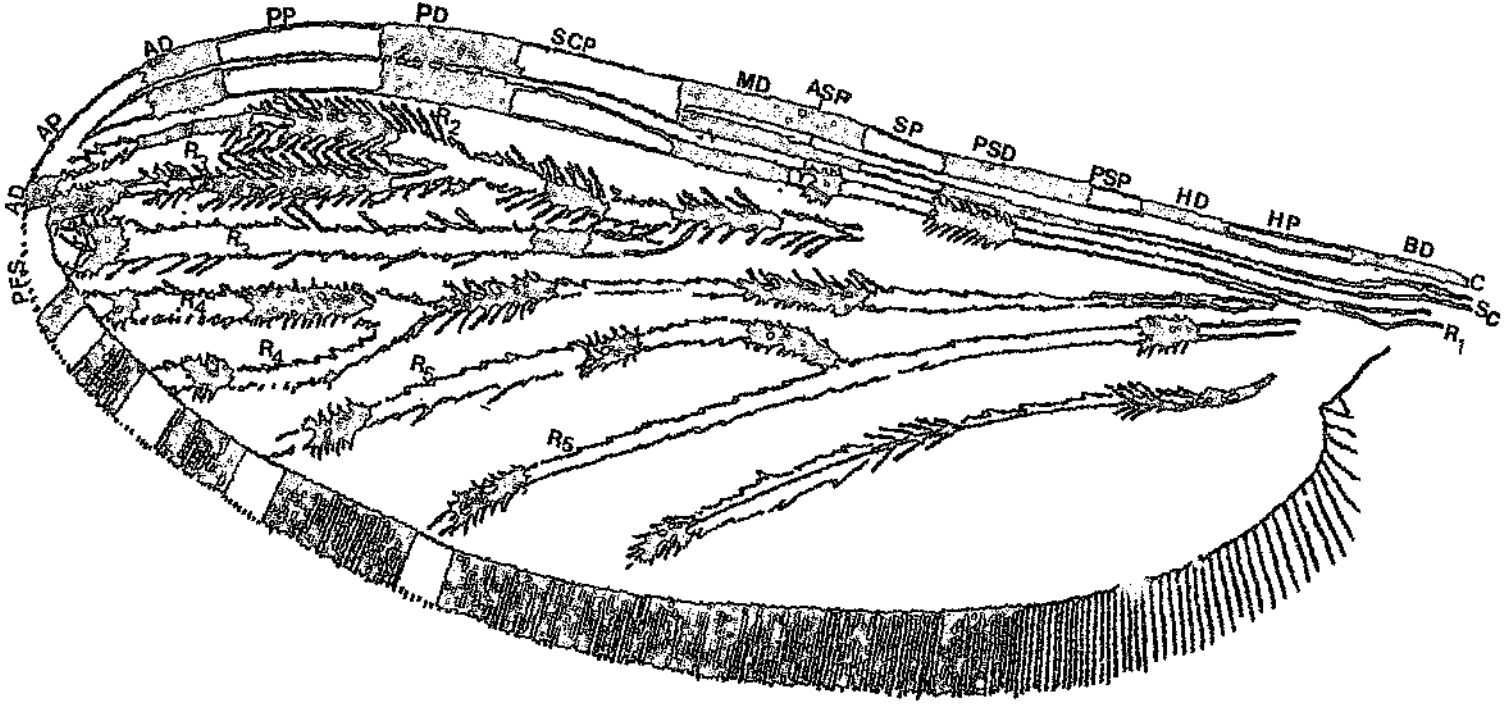

PP
PD
AD
SCP
MD
ASP
AP
SP
PSD
PSP
HD
HP
BD
C
Sc
AD
PFS
R1
R2
R3
R4
R5

TABLE 5

Full setal counts for pupal specimens (n=54) of "*An. listeri* sp.B"

SETA	RANGE
MET	
10	1-4
11	2-4
12	1-3
ABD I	
1	>20
2	4-6
3	2
4	5-6
5	1-3
6	1
7	7-10
9	1
ABD II	
0	1
1	7-8
2	6-8
3	2-3
4	4
5	2-3
6	1
7	3-6
8	2
9	1
10	1-2
ABD III	
0	1
1	6-7
2	5-8
3	1-3
4	3-4
5	6-7
6	3-4
7	2
8	2-4
9	1
10	1-3

SETA	RANGE
ABD III	
11	1
14	1
ABD IV	
0	1
1	1
2	4-5
3	3-7
4	1-3
5	4-6
6	1-3
7	4
8	3-4
9	1
10	1
11	1
14	1
ABDV	
0	1
1	1
2	5-6
3	1-3
4	1-2
5	4-5
6	3-4
7	2-4
8	3-5
9	1
10	1
11	1
14	1
ABD VI	
0	1
1	1
2	5-6
3	1
4	1-2

SETA	RANGE
ABD VI	
5	3-4
6	2-3
7	1-3
8	1-3
9	1
10	1
11	1
14	1
ABD VII	
0	1
1	1
2	3-4
3	1-2
4	1
5	2-3
6	1-4
7	2-3
8	1
9	1
10	1-3
11	1-2
14	1
ABD VIII	
0	1
4	1
9	3-8
14	1
1	1
PADDLE	
1	1
2	1-2

Pupa: Full setal counts (Belkin 1962) are given in Table 5. Differences between "*An. listeri* sp.B" and *An. listeri* (in brackets) are as follows: Seta 1 on abdominal segment II with 7-8 branches (8-10); segment III with 6-7 branches (5-6); segment VII simple and same length as the segment (simple and longer than segment length). Seta 5 on abdominal segments III-VII with 6-7, 4-6, 4-5, 3-4, and 2-3 branches (5-6, 5-6, 3-5, 3-5 and 1-3) respectively. Seta 9 on abdominal segments VI - VII simple and equal to or less than half the segment length (equal to or more than half-length of segment).

Larva: Differences between "*An. listeri* sp.B" and *An. listeri* (in brackets) are as follows: *Antennal Seta*: seta 4 is fine with 3-5 (3-4) branches. *Shoulder Hairs*: inner and median hairs are branched and on small, separate tubercles; inner 9-10 branches (4-5) and median 8-10 branches (9-10). *Palmate Setae*: second abdominal rudimentary and leaflets are lanceolate and undifferentiated with 16(13) branches; third to seventh abdominal fully developed with 16(13) moderately broad leaflets, well defined shoulders and short, blunt-tipped filaments varying in length (De Meillon 1931b). A partial setal count for the head and prothorax on slides of larval specimens can be found on Table 6.

Egg: The shiny, silver-grey egg is boat-shaped (Fig. 5a) with a broad upper surface (deck) The well-developed floats surround approximately two-thirds of the uninterrupted frill. There are 16 float chambers on either side of the egg giving a total of 32 float chambers. A continuous, well-developed, narrow and striated frill (Fig. 5a) surrounds the undivided deck. There are darker, irregular patches

TABLE 6

Partial setal count of head and prothorax for larval specimens (n=42) of "*An. listeri* sp.B"

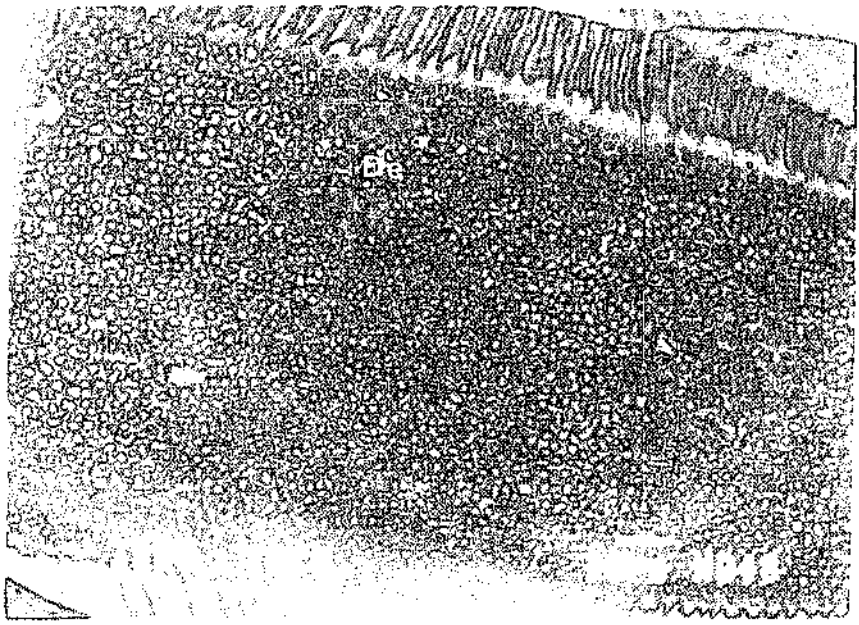
SETA	RANGE
HEAD	
2	1
3	1
4	1
5	7-9
6	3
7	3-4
8	5-8
9	9-12
10	14-17
11	14-18
12	1-3
13	3-5
ANTENNA	
2	1
3	1
4	3-5
PROTHORAX	
1	9-10
2	8-10
3	1

FIGURE 9

Scanning electron micrographs of the eggs of "*An. listeri* sp.B" Botswana

a: Mid, upper-surface showing deck ornamentation (Mag. x 800). De (deck).

b: Anterior and posterior ends showing lobed tubercles (LoT)(Mag. x 2500).



b

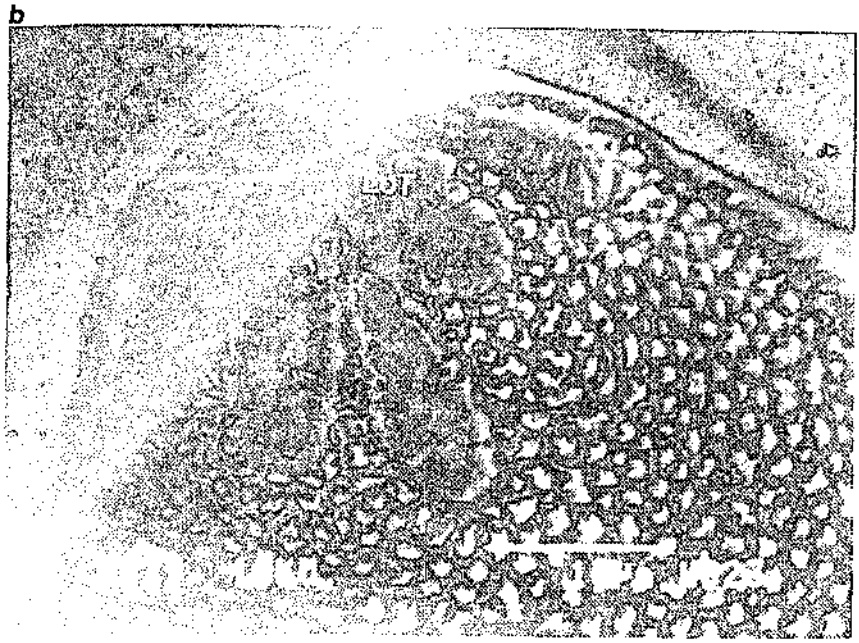


FIGURE 10

Polytene chromosomes from the ovarian nurse cells of *"An. listeri sp.B"* showing Arms X, 4(3R), 5(3L).

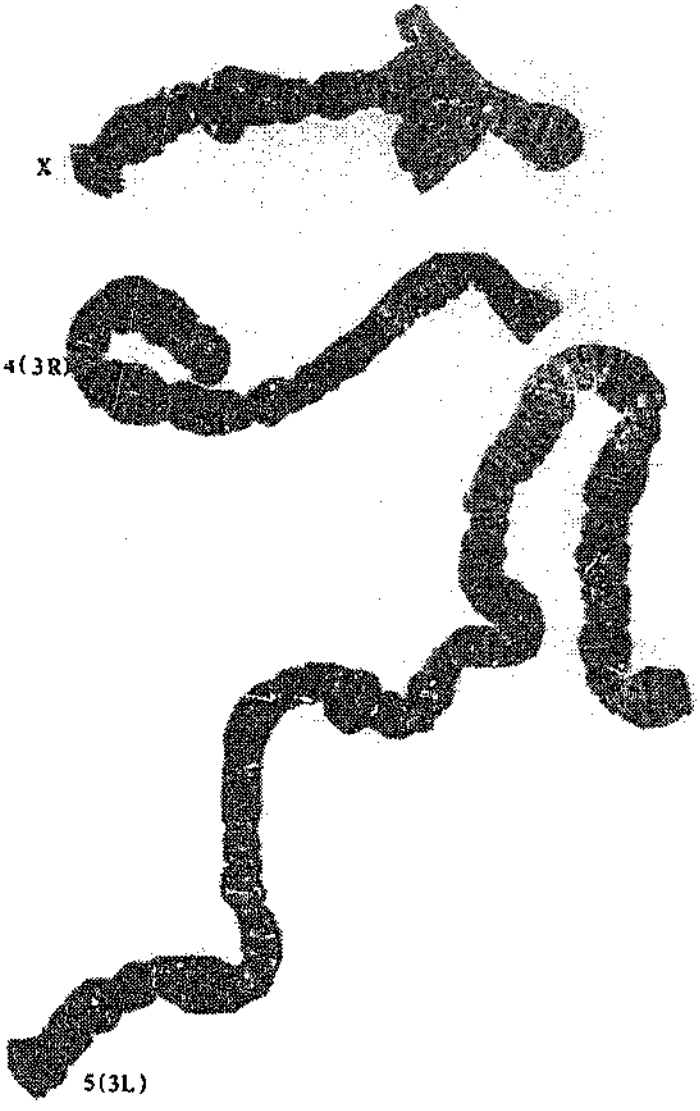
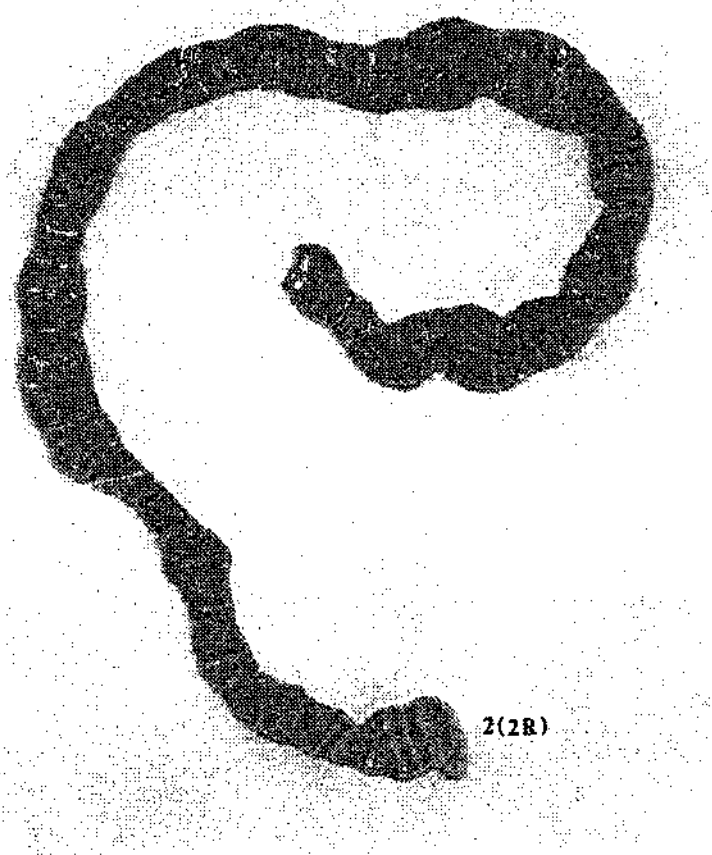

X
4(3R)
5(3L)

FIGURE 11

Polytene chromosomes from the ovarian nurse cells of *"An. listeri sp.B"* showing Arm 2(2R).


2(2R)

(Table 8) setae suggests that setae where no overlap is observed between the two species (setae 10 and 11 on the head of the larva; abdominal setae I-2 and II-7) may be used to separate the two species *An. listeri* Botswana and "*An. listeri* sp.B" in morphological identifications.

The chromosomes of "*An. listeri* sp.B" were found to be homosequential with those of the *An. listeri* chromosomes published in Clements (1992). The quality of the chromosomes of "*An. listeri* sp.B" were good enough to allow comparison with *An. listeri* with respect to arms X, 2, 4 and 5. However, those of *An. listeri* Botswana were not, and only partial homology could be found. This aspect of the study needs to be repeated in order to obtain more preparations and assess any polymorphisms that may occur in the populations.

TABLE 7

COMPARISON OF SELECTED LARVAL SETAE: *AN. LISTERI*, BOTSWANA (N=29) AND "*AN. LISTERI* SP.B"(N=42)

Area of difference		Range per segment **pair** of setae		
Region	**SETA**	**"*An. listeri sp.B*"**	***An. listeri*, Botswana**	**% Overlap**
HEAD	- 9	9-12	11-14	9.86
	- 10	14-17	6-12	0
	- 11	14-18	21-29	0
ANTENNA	- 4	7-12	12-16	15.49

NOTE: Only 71 of the 89 mounted specimens showed most sections of larvae clearly.

TABLE 8

COMPARISON OF SELECTED PUPAL SETAE: *AN. LISTERI*, BOTSWANA (N=34) AND "*AN. LISTERI* SP.B" (N=54)

Area of difference		Range per segment **pair** of setae		
ABD	**SETA**	**"*An. listeri* sp.B"**	***An. listeri*, Botswana**	**% Overlap**
I	- 2	9-11	4-8	0
I	- 3	2-4	2	2.17
II	- 7	7-11	12-17	0
IV	- 8	6-8	2-6	5.43
V	- 8	6-10	2-6	10.87
VIII	- 9	9-16	16-28	9.78

NOTE: Only 88 of the 89 mounted specimens showed most sections of pupae clearly.

CHAPTER 5

THE *ANOPHELES GAMBIAE* COMPLEX

5.1 INTRODUCTION

In Africa, the *An. gambiae* complex of mosquitoes includes major vectors of diseases such as filariasis and malaria. The correct identification of the six members of this important group is imperative in all malarious areas. The currently accepted methods for the identification of members of the *An. gambiae* complex include cytogenetic, isoenzyme and polymerase chain reaction (PCR) analysis. Extensive sampling has shown that cytogenetic analysis has been consistently reliable and is commonly regarded as the "gold standard" for identification. Diagnostic electromorphs have also provided reliable identification providing that gene frequencies are known for the species occurring in a given area. Variation of isoenzymes can also be used as markers in population genetic studies. More recently, PCR analysis provides a rapid and accurate method for the routine identification of members of this complex.

The last documented work carried out in Botswana (Gillies and De Meillon 1968) involved the morphological identification of what is now known as the *An. gambiae* complex. In the present study, 449 morphologically identified *An. gambiae* complex female mosquitoes were collected and transported to the S.A.I.M.R. Insectary. Most laid eggs and a total of 622 F_1 progeny were reared.

Nin. wild larvae were also collected and transported back, but with limited success due to the long distances over which they had to travel.

The samples were subjected to chromosomal, isoenzyme and PCR analyses (Table 9), with particular care being taken to correlate the identifications in order to ensure the accurate identification of vectors in a country where little or no work has been carried out in recent years.

5.2 RESULTS OF THE CHROMOSOMAL ANALYSIS

The chromosomal banding patterns of the *An. gambiae* complex specimens collected from the various localities revealed the presence of only one member of the group, i.e. *An. arabiensis*. Chromosomal rearrangements were recorded for each arm in 252 specimens. The chromosome arm designations and inversion notations used follow those of Coluzzi and Sabatini (1967, 1968, and 1969). Arm 2R had one polymorphic inversion, 2Rb, which is commonly found in southern African populations of *An. arabiensis*. Hardy-Weinberg equilibrium analysis of the pooled data (Table 10) for this arm gives the following results:

	$2Rb^+$	$2Rb/b^+$	$2Rb$
Observed	31	98	123
Expected	25.4	109.2	117.4

Expected frequencies derived from Hardy-Weinberg E = 1.0

$X^2 = 2.651$ DF = 1 NS

When the data for each locality was analysed (where sample size permitted) the following results were obtained for:

TABLE 9

IDENTIFICATION OF SPECIMENS OF THE *AN. GAMBIAE* COMPLEX COLLECTED IN NORTHERN BOTSWANA

LOCALITY	YEAR	CHROMOSOMES	PCR	ISOENZYMES
Maun	1994	39	39	39
Seronga	1994	}	2	0
Kauxwi	1994	}	67	0
Mohembo	1994	} 101	56	0
Shakawe	1994	}	110	0
Shakawe	1995	44	63	0
Kasane	1995	68	267	18

TABLE 10

CHROMOSOMAL IDENTIFICATION OF *AN. GAMBIAE* COMPLEX SPECIMENS FROM NORTHERN BOTSWANA. (n = 252).

CHROMOSOMAL ARRANGEMENTS								No's showing arrangement
2b	2b+		3a	3a+		4a	4a+	X	
1	1			2			2	*arabiensis*	83
	2			2			2	*arabiensis*	24
2				2			2	*arabiensis*	99
	2			2		1	1	*arabiensis*	7
1	1			2		1	1	*arabiensis*	15
2				2		1	1	*arabiensis*	24

NOTE: 11 specimen tubes dried out; chromosomes could not be read.

Maun 1994:

	$2Rb^+$	$2Rb/b^+$	2Rb
Observed	5	19	15
Expected	5.382	18.213	15.366

Expected frequencies derived from Hardy-Weinberg E = 1.0

$X^2 = 0.0697$ DF = 1 NS

Shakawe 1994/1995 (pooled):

	$2Rb^+$	$2Rb/b^+$	2Rb
Observed	18	57	70
Expected	14.94	63.22	66.85

Expected frequencies derived from Hardy-Weinberg E = 1.0

$X^2 = 1.387$ DF = 1 NS

Kasane 1995:

	$2Rb^+$	$2Rb/b^+$	2Rb
Observed	9	32	27
Expected	9.18	31.62	27.132

Expected frequencies derived from Hardy-Weinberg E = 1.0

$X^2 = 0.0092$ DF = 1 NS

When the data for Shakawe were individually analysed the following results were obtained:

Shakawe 1994:

	$2Rb^+$	$2Rb/b^+$	2Rb
Observed	12	36	53
Expected	8.91	42.18	49.92

Expected frequencies derived from Hardy-Weinberg E = 1.0

$X^2 = 2.167$ DF = 1 NS

Shakawe 1995:

	$2Rb^+$	$2Rb/b^+$	2Rb
Observed	6	21	17
Expected	6.204	20.64	17.2

Expected frequencies derived from Hardy-Weinberg E = 1.0

$X^2 = 0.015$ DF = 1 NS

A Hardy-Weinberg analysis of each of the four populations (using one degree of freedom) showed that the deviations were not significant i.e. the observed frequencies of 2Rb+, 2Rb/b+ and 2Rb did not differ significantly from the expected frequencies.

Arm 2L was found to be uniformly monomorphic for the 2La arrangement, which is standard for *An. arabiensis*. Arm 3R was polymorphic for inversion 3Ra with the following frequencies: $3Ra^+$ (n=206), $3Ra/a^+$ (n=46), 3Ra (n=0). The lack of 3Ra homozygotes could be explained by the small sample size or, this homozygote being at a selective disadvantage, or, a balanced polymorphism resulting in the presence of a lethal gene being homozygous in the 3Ra arrangement. However, this is all speculation until larger sample sizes can be analysed.

5.3 PCR IDENTIFICATIONS

The PCR analysis was carried out according to the protocol of Van

FIGURE 12

Photographs showing PCR gels.

a: Colony material (S.A.I.M.R.)

Bands produced by rDNA PCR amplification of DNA from three different species in the *An. gambiae* complex.

An. arabiensis (1,4,5,6,8,10,12,13).

An. gambiae (2,3,7,9,14,15,16).

1 kb ladder (11).

Standards: *An. arabiensis* (17), *An. gambiae* (18), *An. merus* (19).

b: Bands produced by rDNA amplification of DNA from legs and terminal segments of material collected in Botswana (*An. arabiensis*).

1 kb ladder (1,18).

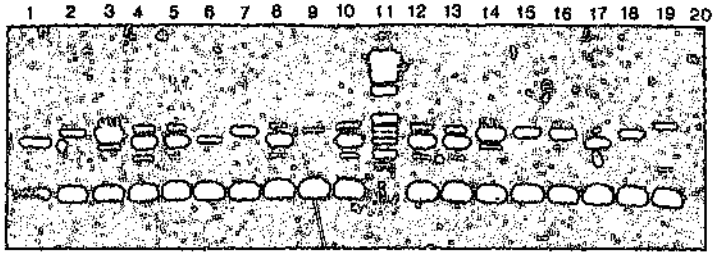
An. arabiensis (2,3,4,5,6,7,8,9,10,11,12,13); legs.

No PCR band (14).

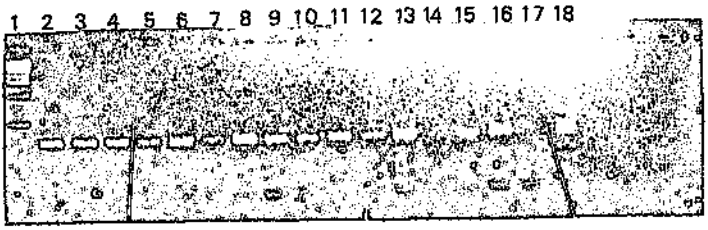
An. arabiensis (15,16); terminal segments.

Negative control (17).

a


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

b


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Rensburg *et al*, (1996) (see Appendix II). A total of 622 specimens were tested and 604 were identified as *An. arabiensis* (Fig. 12b, Table 9). Eighteen specimens produced no bands because the alcohol in the tubes had dried out and the DNA template was probably degraded. Of the 604 PCR identified specimens, 252 were chromosomally correlated (Table 9). The PCR analysis enables straightforward reliable identifications: previous tests on colony material were found to be 100% accurate (Van Rensburg *et al.*, 1996). PCR also proved to be of particular use as little was known about the species composition of the *An. gambiae* complex in Botswana when this study was initiated.

5.4 ISOENZYME ANALYSIS

Eighteen *An. gambiae* complex samples from Shakawe (1994) were identified using a flat bed horizontal cooling system as outlined in Harris and Hopkinson (1976). All other isoenzyme identifications were carried out using a modified version of the vertical gel electrophoretic technique outlined in Munstermann (pers.comm., unpublished laboratory notes) (see Appendix I). The enzyme systems were run on polyacrylamide gels and stained according to the methods of Mahon *et al.* (1976) and Miles (1978) (see Appendix I). The stained gels were then dried, mounted and copied (Figs. 13-16).

The isoenzyme data were analysed using BIOSYS-I, a computer program for the analysis of allelic variation in population genetics (Swofford and Selander 1989). BIOSYS-I generated (i) the extent of polymorphism; (ii) observed and expected heterozygosities and (iii) Wright's (1978) F-statistics. Since *Nm* calculations were not available in BIOSYS-I, estimates of *Nm* were derived from

FIGURE 13

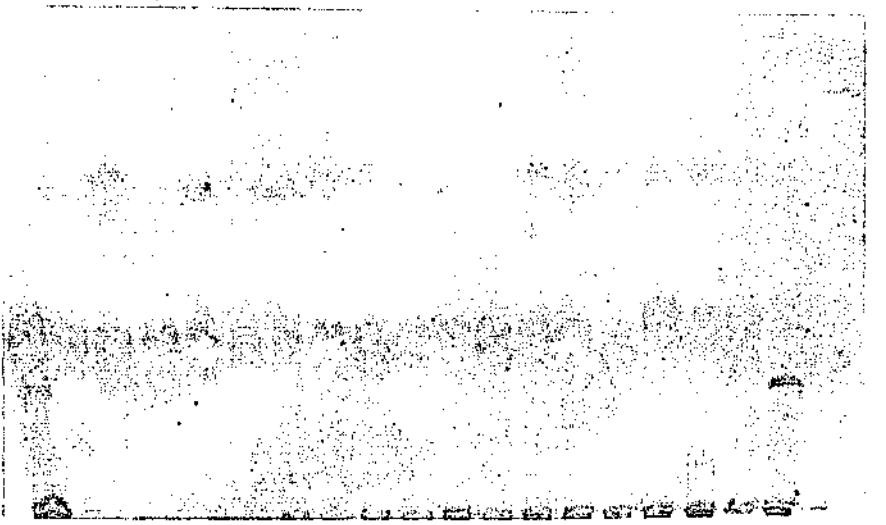
Maun 1994:

a: Polyacrylamide gel stained for EST.
Lane 1 (H) Human blood marker
Lane 2 *An. arabiensis* control
Lane 20 *An* ontrol

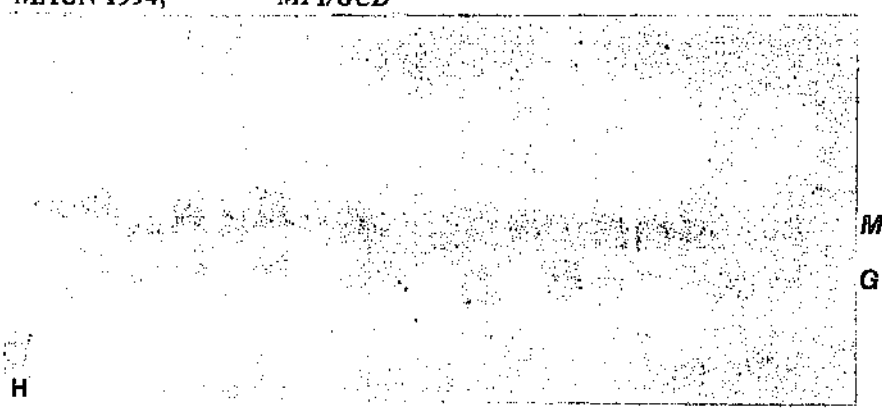
b: Polyacrylam s stained for MPI/GCD.
Lane 1 (H) Human blood marker
Lane 2 *An. arabiensis* control
Lane 20 *An. merus* control

c: Polyacrylamide gel stained for XDH
Lane 1 (H) Human blood marker
Lane 2 *An. arabiensis* control

MAUN 1994; *EST*



MAUN 1994; *MPI/GCD*

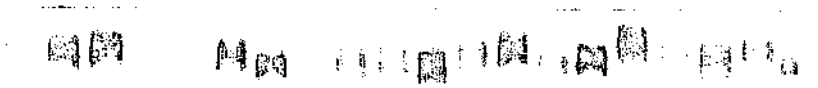


MPI

GCD

H

MAUN 1994; *XDH*



H

FIGURE 14

Shakawe 1994:

a: Polyacrylamide gel stained for ODH

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

b: Polyacrylamide gel stained for AAT

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

c: Polyacrylamide gel stained for XDH

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

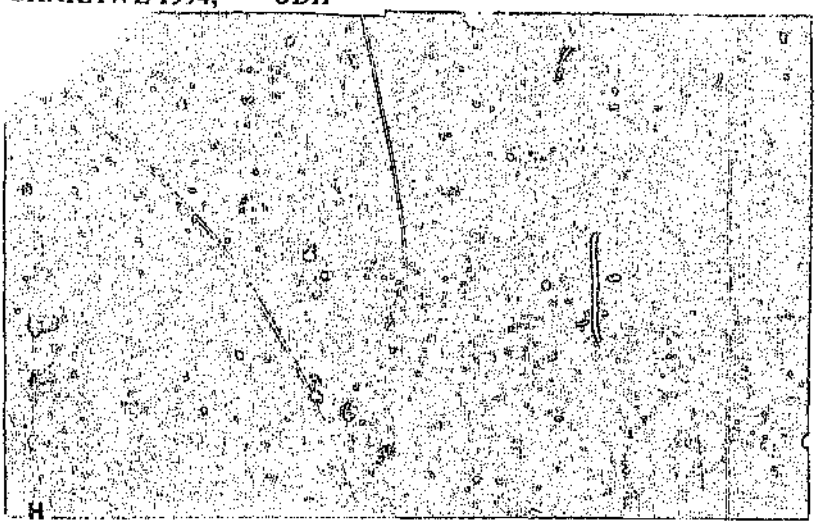
d: Polyacrylamide gel stained for MPI/GCD

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

Lane 9 *An. merus* control

SHAKAWE 1994; ***ODH***



H

SHAKAWE 1994; ***AAT***

H

SHAKAWE 1994; ***XDH***

H

SHAKAWE 1994; ***MPI/GCD***

MPI

GCD

H

FIGURE 15

Shakawe 1995:

a: Polyacrylamide gel stained for EST

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

Lane 19 *An. merus* control

b: Polyacrylamide gel stained for MPI/GCD

Lane 1 (H) Human blood marker

Lane 2 (*An. arabiensis* control

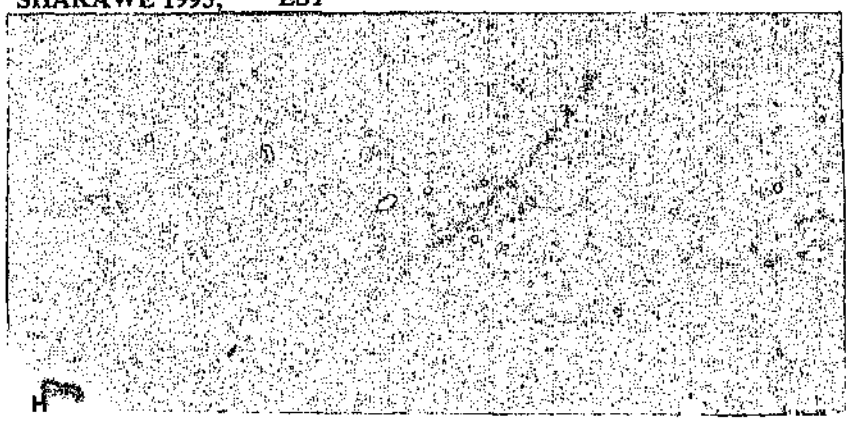
c: Polyacrylamide gel stained for AAT

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

Lane 12 ?

SHAKAWE 1995; ***EST***



H

SHAKAWE 1995; ***MPI/GCD***

MPI

GCD

H

SHAKAWE 1995; ***AAT***

H

FIGURE 16

Kasane 1995:

a: Polyacrylamide gel stained for EST

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

Lane 20 *An. merus* control

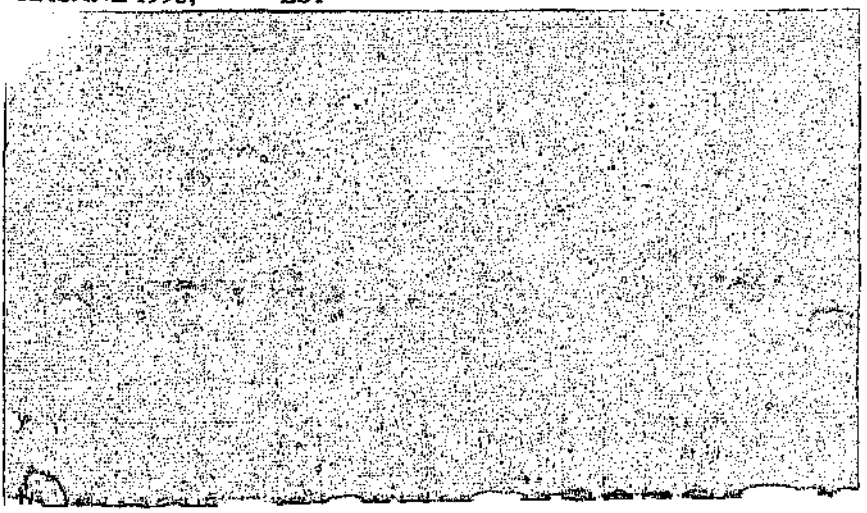
b: Polyacrylamide gel stained for MPI/GCD

Lane 1 (H) Human blood marker

Lane 2 *An. arabiensis* control

Lane 20 *An. merus* control.

KASANE 1995; *EST*



KASANE 1995; *MPI/GCD*

H

F_{ST} values for each of the six loci in all four populations according to Slatkin (1995): $Nm = ¼ (1/F_{ST} - 1)$.

Gene frequencies at each of the six isoenzyme loci studied in the four populations of *An. arabiensis* sampled are given in Tables 11-16. Heterozygosity values for each population are given in Table 17.

The Hardy-Weinberg Law: This law states that in a large, randomly-mating population with no selection, mutation or migration, the gene frequencies and the genotype frequencies are constant from generation to generation. There will, of course, be gene flow within the population as a result of mating but the overall gene frequencies remain constant (Lewontin 1974, Li 1976, Hartl 1988). This stability is referred to as genetic equilibrium. Under these circumstances a deme (more or less genetically isolated unit of population) remains unchanged in its overall characteristics and no evolution occurs. The Hardy-Weinberg Law states that if the gene frequencies among the parents are p and q respectively, then the genotype frequencies among the progeny will be $p^2 + 2pq + q^2$ for autosomal genes. However, there are certain conditions that need to be observed in order for the Law to hold, namely (i) random mating must occur; (ii) there must be a normal segregation of genes in gametogenesis; (iii) no selection should take place and (iv) gene frequencies must be the same for males and females (Lewontin 1974, Li 1976). This law, though useful as a first approximation, ignores most of the complexities of actual populations (Hartl 1988). Since populations are not infinitely large, and, rarely constant, fluctuations in allele frequencies can occur by

TABLE 11

ALLELE FREQUENCIES AT SIX ISOENZYME LOCI IN *AN. ARABIENSIS* FROM FOUR LOCALITIES IN NORTHERN BOTSWANA

KEY TO POPULATIONS

Population No. On printout	Population Name	Original Population No.
1	Maun 1994	(DS1)
2	Shakawe 1994	(DS2)
3	Shakawe 1995	(DS3)
4	Kasane 1995	(DS4)

Allele frequencies in populations 1 through 4.

Locus	POPULATION			
	1	2	3	4
EST				
(N)	39	53	63	288
A	.077	.047	.048	.068
B	.192	.123	.286	.241
C	.692	.774	.571	.590
D	.038	.057	.095	.101
GCD				
(N)	39	36	62	288
A	.038	.056	.056	.076
B	.128	.069	.169	.231
C	.731	.736	.702	.620
D	.103	.139	.073	.069
E	.000	.000	.000	.003
AAT				
(N)	39	53	63	289
A	000	.038	.000	.024
B	.038	.094	.111	.240
C	.923	.755	.770	.661
D	.038	.113	.119	.074
ODH				
(N)	39	53	63	289
A	.000	.009	.000	.059
B	.936	.991	.952	.919
C	.064	.000	.048	.022
MPI				
(N)	39	35	62	288
A	.372	.029	.226	.148
B	.564	.829	.694	.785
C	.013	.114	.016	.036
D	.051	.029	.065	.024
E	.000	.000	.000	.007
XDH				
(N)	39	36	63	287
A	.038	.000	.024	.005
B	.051	.125	.310	.157
C	.333	.764	.556	.605
D	.564	.097	.103	.220
E	.013	.014	.008	.014

TABLE 12 MEAN HETEROZYGOSITY, MEAN NO. OF ALLELES PER LOCUS AND PERCENTAGE OF LOCI POLYMORPHIC FOR POPULATIONS 1 THROUGH 4

	POPULATION NUMBER			
	DS1	DS2	DS3	DS4
Mean heterozygosity per locus (biased)	381 (SE .081)	.321 (.063)	.482 (.075)	.451 (.068)
Mean heterozygosity per locus (unbiased)	.386 (SE .082)	.325 (.064)	.432 (.075)	.451 (.068)
Mean heterozygosity per locus (direct count)	.256 (SE .068)	.239 (.056)	.285 (.053)	.331 (.037)
Mean number of alleles per locus	3.67 (SE .42)	3 67 (.33)	3 67 (.42)	4 33 (.33)
Percentage of loci polymorphic (0.95 criterion)	100	83 33	83.33	100
Percentage of loci polymorphic (0.99 criterion)	100	83 33	100	100
Percentage of loci polymorphic (no criterion)	100	100	100	100

chance. Natural populations are also subject to the systematic evolutionary forces of migration, mutation and natural selection (Li 1976, Hartl 1988, Hartl and Clark 1989, Falconer and Mackay 1996) all of which cause non-random or directional changes in allele frequency.

Deviations from Hardy-Weinberg expectations in the Botswana sample:

Heterozygosity is the most appropriate measure of the genetic variability of a population (Nei 1975). If a population is in Hardy-Weinberg equilibrium, the observed heterozygotes must equal the expected heterozygotes. An analysis of the genetic variability at six loci in all four Botswana populations showed heterozygote deficiencies (at the 0.01 significance level) in each of the four populations: **Maun** 94 (Table 13) MPI; **Shakawe** 94 EST and GCD (Table 14); **Shakawe** 95 EST, GCD and XDH (Table 15); **Kasane** 95 EST, GCD, AAT and XDH (Table 16). Coefficients for heterozygote deficiency in the four populations are given in Table 17. These results suggest that the Botswana population is a subdivided population.

Population subdivision: Natural populations almost always display differences in allele and genotype frequencies from one geographic region to another. Such geographic population structure can have a profound effect on the evolutionary fate of a species. The effects of population subdivision are measured by a quantity called the Fixation Index (F_{ST}). F_{ST} is the reduction in heterozygosity of a subpopulation and serves as a convenient and widely used measure of genetic differences among different populations. If all the populations are in Hardy-

TABLE 13

CHI-SQUARE TEST WITH POOLING

POPULATION: MAUN 94, BOTSWANA (DS1)

Locus	Class	Observed Frequency	Expected frequency	Chi-square	DF	P	
EST	Homozygotes for most common allele	21	18.584				
	Common/rare heterozygotes	12	16.831				
	Rare homozygotes and other heterozygotes	6	3.584	3.329	1	.068	NS
GCD	Homozygotes for most common allele	21	20.727				
	Common/rare heterozygotes	15	15.545				
	Rare homozygotes and other heterozygotes	3	2.727	.050	1	.823	NS
AAT	Homozygotes for most common allele	33	33.195				
	Common/rare heterozygotes	6	5.610				
	Rare homozygotes and other heterozygotes	0	.195	.223	1	.637	NS
MPI	Homozygotes for most common allele	21	12.286				
	Common/rare heterozygotes	2	19.429				
	Rare homozygotes and other heterozygotes	16	7.286	32.238	1	.000	S
XDH	Homozygotes for most common allele	16	12.286				
	Common/rare heterozygotes	12	19.429				
	Rare homozygotes and other heterozygotes	11	7.286	5.857	1	.016	NS

DF = **Degrees of freedom** **P** = **Probability that variation is due to chance**
NS = **Not significant** **S** = **Significant (p<0.01)**

TABLE 14

CHI-SQUARE TEST WITH POOLING

POPULATION: SHAKAWE 94, BOTSWANA (DS2)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P	
EST	Homozygotes for most common allele	35	31.629				
	Common/rare heterozygotes	12	18.743				
	Rare homozygotes and other heterozygotes	6	2.629	7.109	1	.008	S
GCD	Homozygotes for most common allele	24	19.408				
	Common/rare heterozygotes	5	14.18				
	Rare homozygotes and other heterozygotes	7	2.408	15.785	1	.000	S
AAT	Homozygotes for most common allele	31	30.095				
	Common/rare heterozygotes	18	19.810				
	Rare homozygotes and other heterozygotes	4	3.095	.457	1	.499	NS
MPI	Homozygotes for most common allele	24	23.957				
	Common/rare heterozygotes	10	10.087				
	Rare homozygotes and other heterozygotes	1	.957	.003	1	.958	NS
XDH	Homozygotes for most common allele	21	20.915				
	Common/rare heterozygotes	13	13.169				
	Rare homozygotes and other heterozygotes	2	1.915	.006	1	.937	NS

DF = Degrees of freedom **P** = Probability that variation is due to chance
NS = Not significant **S** = Significant ($p<0.01$)

TABLE 15

CHI-SQUARE TEST WITH POOLING

POPULATION: SHAKAWE 95, BOTSWANA (DS3)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P	
EST	Homozygotes for most common allele	34	20.448				
	Common/rare heterozygotes	4	31.104				
	Rare homozygotes and other heterozygotes	25	11.448	48.643	1	.000	S
GCD	Homozygotes for most common allele	36	30.415				
	Common/rare heterozygotes	15	26.171				
	Rare homozygotes and other heterozygotes	11	5.415	11.555	1	.001	S
AAT	Homozygotes for most common allele	40	37.248				
	Common/rare heterozygotes	17	22.504				
	Rare homozygotes and other heterozygotes	6	3.248	3.881	1	.049	NS
MPI	Homozygotes for most common allele	28	29.715				
	Common/rare heterozygotes	30	26.569				
	Rare homozygotes and other heterozygotes	4	5.715	1.057	1	.304	NS
XDH	Homozygotes for most common allele	27	19.320				
	Common/rare heterozygotes	16	31.360				
	Rare homozygotes and other heterozygotes	20	12.320	15.364	1	.000	S

DF = Degrees of freedom **P** = Probability that variation is due to chance
NS = Not significant **S** = Significant ($p<0.01$)

TABLE 16

CHI-SQUARE TEST WITH POOLING

POPULATION: KASANE 95, BOTSWANA (DS4)

Locus	Class	Observed frequency	Expected frequency	Chi-square	DF	P	
EST	Homozygotes for most common allele	118	100.226				
	Common/rare heterozygotes	101	139.548				
	Rare homozygotes and other heterozygotes	66	48.226	18.758	1	.000	S
GCD	Homozygotes for most common allele	123	110.515				
	Common/rare heterozygotes	111	135.970				
	Rare homozygotes and other heterozygotes	54	41.515	9.751	1	.002	S
ADH	Homozygotes for most common allele	153	126.120				
	Common/rare heterozygotes	76	129.761				
	Rare homozygotes and other heterozygotes	60	33.120	49.819	1	.000	S
MPI	Homozygotes for most common allele	174	177.263				
	Common/rare heterozygotes	104	97.475				
	Rare homozygotes and other heterozygotes	10	13.263	1.299	1	.254	NS
XDH	Homozygotes for most common allele	125	104.766				
	Common/rare heterozygotes	97	137.468				
	Rare homozygotes and other heterozygotes	65	44.766	24.966	1	.000	S

DF = Degrees of freedom **P** = Probability that variation is due to chance

NS = Not significant **S** = Significant ($p<0.01$)

TABLE 17
COEFFICIENTS FOR HETEROZYGOTE DEFICIENCY OR EXCESS

POPULATION: MAUN '94, BOTSWANA (DS1)

LOCUS	OBSERVED HETEROZYGOTES	EXPECTED HETEROZYGOTES	FIXATION INDEX (F)	D
EST	13	18.818	300	-.309
GCD	16	17.2[illegible]	.062	-.074
AAT	6	5.727	-.061	048
ODH	5	4.710	-.068	.055
MPI	2	21.364	.905	-.906
XDH	18	22.377	185	-.196

POPULATION: SHAKAWE' 94, BOTSWANA (DS2)

LOCUS	OBSERVED HETEROZYGOTES	EXPECTED HETEROZYGOTES	FIXATION INDEX (F)	D
EST	14	20.390	307	-.313
GCD	5	15.732	0678	-.682
AAT	13	21.790	166	-.174
ODH	1	1.000	-.010	.000
MPI	10	10.609	.044	-.057
XDH	14	14.282	006	-.020

POPULATION: SHAKAWE' 93, BOTSWANA (DS3)

LOCUS	OBSERVED HETEROZYGOTES	EXPECTED HETEROZYGOTES	FIXATION INDEX (F)	D
EST	14	36.864	.617	-.620
GCD	21	29.415	.280	-.286
AAT	19	24.184	.208	-.214
ODH	6	5.760	-.050	.042
MPI	30	28.976	-.044	.035
XDH	17	37.104	.538	-.542

POPULATION: KASANE' 93, BOTSWANA (DS4)

LOCUS	OBSERVED HETEROZYGOTES	EXPECTED HETEROZYGOTES	FIXATION INDEX (F)	D
EST	118	166.930	292	-.293
GCD	114	159.216	283	-.284
AAT	85	144.536	.411	-.412
ODH	47	4[illegible].919	-.070	068
MPI	106	103.995	-.021	019
XDH	102	161.447	367	-.368

Weinberg equilibrium, with the same allele frequencies i.e. only random mating taking place, $F_{ST} = 0$. Wright's F-statistics (1978) measure the departure from the expectation of panmictic or randomly mating proportions in the whole sample and also within samples of local demes. An interpretation of the F_{ST} values for the Botswana sample (Table 18) was made following the guidelines in Wright (1978). In addition, the estimates of migration (*Nm*) were derived from the F_{ST} values generated in BIOSYS-I (Table 19). An analysis of *Nm* values is useful because it gives an indication of the extent of new variation introduced into populations brought about by some migration between localities.

***Nm* values derived for the Botswana populations:** The estimated number of migrants per generation (*Nm*) calculated from F_{ST} values (Slatkin 1995) for the entire Botswana population ranged between 0.58 and 8.08 (Table 19). *Nm* values less than or approximately equal to 2 observed for the EST, GCD, AAT and MPI loci suggest that there is scope for genetic divergence resulting from random genetic drift (Hartl 1988, Hartl and Clark 1989) in these loci. *Nm* values (8.08) calculated for the ODH locus suggest that ODH could be under strong selective pressure resulting in no divergence between populations or, alternatively, that migration is a potent force acting against genetic divergence resulting from random genetic drift among subpopulations where these findings are supported by the F_{ST} value (Table 18) which suggested little genetic differentiation for that locus in all the populations sampled. *Nm* values (3.32) for the XDH locus suggest that there should be little or no genetic divergence which is contradicted by the findings of the F_{ST} analysis in the Maun 94 and Shakawe 94 populations. Similar

TABLE 18

A summary of Wright's F-statistical analysis on the Botswana sample and the implications of the results obtained for six loci.

			BOTSWANA			
F_{ST} ranges (Wright, 1978)	Genetic Divergence (differentiation)	Gene flow Levels	Maun 94	Shakawe 94	Shakawe 95	Kasane 95
0	None	High			XDH	
0-0.05	Little	High	ODH	ODH	ODH	ODH, XDH
0.05-0.15	Moderate	Fairly High	EST	EST, AAT	GCD	EST, GCD
0.15-0.25	Great	Low	XDH, AAT		EST	AAT
≥0.25	Very great	Very Low	MPI, GCD	GCD, MPI, XDH	MPI, AAT	MPI

TABLE 19

Analysis of F_{ST} and derived estimates of *Nm* for six polymorphic loci from four local populations of *An. gambiae* complex mosquitoes (Botswana).

	F_{ST} VALUES					
LOCUS	MAUN 94	SHAKAWE 94	SHAKAWE 95	KASANE 95	AVERAGE F_{ST}	AVERAGE *Nm* value
EST	0.14	0.13	0.17	0.15	0.15	1.42
GCD	0.29	0.28	0.07	0.07	0.18	1.14
AAT	0.22	0.12	0.27	0.24	0.21	0.94
ODH	0.03	0.03	0.01	0.03	0.03	8.08
MPI	0.27	0.3	0.3	0.34	0.30	0.58
XDH	0.24	0.37	-0.35	0.02	0.07	3.32

The F_{ST} values are the averages for a locus of the values computed for each allele.
The estimate of *Nm* is based on Wright's formula (Slatkin, 1995).
$Nm = {}^1/_4 ({}^1/F_{ST} - 1)$.

contradictory results were obtained from two separate studies on *Aedes aegypti* where Powell *et al.* (1980) showed that this species, with respect to F_{ST} values, has a complex structure with substantial genetic differentiation even though extensive gene flow (Nm>9) was documented across approximately 150 km in Puerto Rico by Apostol *et al.* (1995).

5.5 DISCUSSION

An understanding of the genetic structure of *An. gambiae* complex populations is critical in evaluating the possibility of the genetic manipulation of this species in order to either block malaria transmission, or, to aid in the control of vectors based on currently available technology. The importance of species-specific enzyme protein phenotypes in studies of the *An. gambiae* complex has been discussed by Mahon *et al.* (1976). Identifications based on diagnostic allozymes for example those at EST-1 (*An. melas*), AAT (*An. quadriannulatus*) and SOD (*An. bwambae, An. merus*) can be made regardless of sex and stage of the gonotrophic cycle. An extensive study on the enzyme variation in the *An. gambiae* complex was carried out by Miles (1978) in a number of different localities. The allele frequencies were calculated, though no attempt was made to analyse them. This allele frequency data was later utilised by Lehmann *et al.* (1996).

A brief comparison will be made here between the Botswana study and one similar to it carried out by Lehmann *et al.* (1996). Lehmann *et al.* (1996) analysed the genetic variation of *An. gambiae* to assess the interpopulation divergence over 6000 kilometers using five polymorphic microsatellite loci and six allozyme loci.

The frequencies of the six allozyme loci used in their study were obtained from Miles (1978).

F-statistics were calculated based on Wright (1978) for microsatellite and allozyme data using the BIOSYS I statistical computer analysis programme. These calculations showed low estimates of interpopulation differentiation which corresponded to high estimates of the average migration index ($Nm > 3$) suggesting that gene flow across the continent is only weakly restricted. Active dispersal was ruled out as being a possible cause for these results as mark – release – capture experiments (Gillies and De Meillon 1968) have suggested that active dispersal in *An. gambiae* is restricted to a few kilometers. In contrast with the low levels of differentiation observed in *An. gambiae* populations in the Lehmann *et al.* (1996) study, the Botswana *An. arabiensis* populations showed varying levels of genetic differentiation (Tables 18,19) across a smaller geographical range when subjected to F-statistical analysis using BIOSYS I. The results of the Botswana study would seem to be more compatible with the high levels of chromosomal – inversion – based genetic population structure recorded in *An. gambiae* populations particularly from East and West Africa (Coluzzi *et al.*, 1979, Petrarca *et al.*, 1987).

At this point, therefore, there seems to be insufficient information to explain either the low differentiation of *An. gambiae* populations (Lehmann *et al.*, 1996) across Africa over a distance of 6000 kilometers or, the varying levels of genetic differentiation seen in the *An. arabiensis* populations over much smaller geographical distances within Botswana. Information based on additional genetic loci, different genetic markers, and, populations on different geographical scales is

needed before any final conclusions can be reached on the nature of the Botswana *An. arabiensis* populations.

CHAPTER 6

DISCUSSION

6.1 SPECIES CONC[illegible] - INTRODUCTION

The immense diversity of species in nature can not be discussed without mentioning both classification and nomenclature. Aristotle, popularly known as the originator of biological classification, suggested the existence of "higher" and "lower" forms of life, according to how "perfect" these were; an idea that was subsequently translated [illegible]volutionary terms (Mayr, 1969). Linnaeus introduced the binomial method of nomenclature making use of Aristotle's system of logic in his classification. The system was based on visible morphological differences and the idea that species were divine creations. Since Linnaeus' ideas on taxonomy were based on data that were limited to observable characteristics, they cannot justifiably be considered as a "species concept". Today, the conceptual distinctness of, and the relationship between "genetical species" and "taxonomic species" are generally accepted by population biologists.

There are currently two rival concepts of species in genetical terms. Most population biologists agree that species are real biological entities, however, there exist two schools of thought regarding the genetical nature of species and how they arise. These rival concepts are (i) the "Isolation Concept" and (ii) the "Recognition Concept". Both will be described briefly in an endeavour to indicate the reasons for my preference of the Recognition Concept.

6.2 THE "*LISTERI*" GROUP OF MOSQUITOES IN BOTSWANA

Just how "species" are recognised in the anophelines, and what implications chromosomally homosequential species like *An. listeri* De Meillon, and "*An. listeri* sp.B" have for such recognition is a matter of debate. The fact that both species were found to share the same X chromosome arrangement is contrary to, for example, Kitzmiller's (1977) expectations. In his review of anopheline cytology, he states that in most cases the X chromosomes are distinctive enough to permit species identification on their own and then goes on to quote the *An. gambiae* complex as an example of fixed inversion differences on the X marking species differences in *An. gambiae s.s.*, *An. arabiensis* and *An. quadriannulatus*. By saying this, he ignores a previous statement in which he mentions that three other species of the group share two of these X arrangements: *An. merus* and *An. gambiae* share one arrangement which differs by one inversion from the X shared by *An. melas*, *An. quadriannulatus* and *An. bwambae* which differs by a further three inversions from *An. arabiensis*. Placing total dependence on chromosomal arrangements as species markers would put cytologists on an equal footing with a taxonomist who equates pigeon-holes with biological species.

It has been suggested by M.J.D. White (1973) and G.B. White (1973) that in anopheline speciation, chromosomal rearrangements may be a primary cause of speciation giving weight to the idea that such rearrangements bear a causal relationship to speciation. This idea suggests that chromosomal rearrangements should be expected to serve as very good species markers. However, neither of these authors offer any convincing evidence which would warrant abandoning the

view that chromosomal rearrangements should be seen as coincidental, chance events and not causal of the speciation process.

6.3 THE *ANOPHELES ARABIENSIS* MOSQUITOES FROM BOTSWANA

Collections of the *An. gambiae* complex of mosquitoes from Botswana which were subjected to morphological, cytogenetic, isoenzyme electrophoretic and PCR analyses revealed the presence of only *An. arabiensis*. According to a paper presented at a regional malaria conference (Francistown, 1995), malaria in Botswana was predominantly transmitted by *An. gambiae*, *An. arabiensis* and *An. funestus*. Also stated in the paper was that three members of the *An. gambiae* complex had been identified (*An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus*) with *An. gambiae s.s.* and *An. arabiensis* occurring in sympatry throughout the Northwest District, Tutume and Francistown and *An. quadriannulatus* occurring in Nata, Shakawe and Kazangula (Figs. 1,2). Our four-year study, which overlapped with the study period mentioned in the paper presented at this Conference, confirmed the presence of *An. arabiensis* only in the same areas of northern Botswana. These conflicting reports only serve to reinforce the importance of correct identification of vector species in any malaria control programme. The results of this study suggest that *An. arabiensis* and, to a lesser extent, *An. funestus* are the vector species in Botswana.

Electrophoresis of the Botswana sample was initially carried out in order to identify the specimens and was not intended to be used as a tool for measuring isoenzyme variability, hence the limited number of loci used in the analysis. Statistical analyses of genetic variability at six loci in all four populations showed

heterozygote deficiencies in: MPI locus (Maun 94); EST and GCD loci (Shakawe 94); EST, GCD and XDH loci (Shakawe 95) and EST, GCD, AAT and XDH loci (Kasane 1995). These suggest that the Botswana population is subdivided.

Southern African *An. arabiensis* is usually monomorphic for ODH-95. The Botswana sample was monomorphic for ODH-95 in most specimens with a small minority of samples showing ODH 95/100. This is in contrast with a Namibian sample (Coetzee, 1996) which displayed the ODH-100 allele frequently. ODH-100 is diagnostic for *An. gambiae s.s.* showing little overlap with *An. arabiensis* in East and southern Africa (Miles 1979). Miles (1978) recorded the greatest amount of variation in *An. arabiensis* from the Cameroon, Nigeria and Senegal where ODH-100 was common leading to a misidentification of the species in as many as 7% of the specimens tested.

The aspartate aminotransferase (AAT) isoenzyme system was initially utilised by Miles (1978, 1979) to separate populations of *An. quadriannulatus* from other members of the *An. gambiae* complex. The *An. quadriannulatus* bands were "slow" and arbitrarily designated "95". All other members of the *An. gambiae* complex were "fast" and designated "100". In this study, resolution of the bands was improved by changing to the staining system of L.E. Munstermann (pers. comm., unpublished laboratory notes). Heterozygotes were recognised with ease. In South Africa, Coetzee *et al.* (1993a) established that *An. quadriannulatus* was highly polymorphic, though always migrating slower than the "95" designation, while the other *gambiae* complex members were only rarely polymorphic with a mobility of either "100" or faster. AAT was highly polymorphic in the Namibian sample of *An. arabiensis* and overlapped with the

diagnostic *An. quadriannulatus* electromorph (Coetzee *et al.*, 1993a). The variation found in *An. arabiensis* from Namibia was unexpected. If we consider Coetzee's (1996) hypothesis that Namibian *An. arabiensis* may well be a different species to South African *An. arabiensis*, then using AAT as the marker, it could be postulated that the Namibian *An. arabiensis* spreads eastwards across Botswana to Kasane and the South African *An. arabiensis* spreads westwards to Kasane where they meet but do not mate. This would account for the massive deficiency of heterozygotes (Table 16) in the Kasane *An. arabiensis* which was highly monomorphic.

The possibility that the taxon *An. arabiensis* may consist of more than one species becomes more acceptable in the light of the chromosomal variation recorded in West Africa (Coluzzi *et al.*, 1979, Petrarca *et al.*, 1987) together with the frequently recorded differences in behaviour. Coluzzi *et al* (1979) recorded marked geographical differences in both the distribution and the frequency of inversion polymorphisms in East and West African populations of *An. arabiensis*. Inversion 2Ra for example, occurred in populations from Senegal to the Sudan, but was never reported for southern savanna populations. Arm 2R in the Botswana population had one floating inversion, 2Rb, which is commonly found in southern African populations. In West African forest town samples, 2Ra was absent and 2Rb fixed, which is in contrast with savanna populations in West Africa, but similar to those in East Africa (Coluzzi *et al.*, 1979). Arm 2L in the Botswana sample was uniformly monomorphic for 2La arrangement, which is standard for *An. arabiensis*. Arm 3R was polymorphic for inversion 3Ra with the frequencies: 3Ra (n=206), 3Ra/a+ (n=46), 3Ra (n=0). The lack of 3Ra

homozygotes in the Botswana population was either due to the fact that the sample size was small, or, this homozygote was at a selective disadvantage, or, a balanced polymorphism resulted in the presence of a lethal gene being homozygous in the 3Ra arrangement.

The possibility that two species may share the same or extremely similar DNA sequences has already been demonstrated for the diagnostic PCR bands in (i) East African *An. merus* and West African *An. melas* (Scott *et al.*, 1993) and (ii) *An. merus* and *An. quadriannulatus* (Van Rensburg *et al.*, 1996). The method utilised by Scott *et al.* (1993) cannot, therefore, be expected to provide additional insight into the specific status of *An. arabiensis* populations.

Additional insights into the specific status of *An. arabiensis* populations may be provided by microsatellite DNA markers (Zheng, *et al.*, 1993, Lanzaro *et al.*, 1995), mitochondrial DNA, RFLP's and RAPD's. This work remains to be carried out for the Botswana and other African samples of *An. arabiensis*.

APPENDIX I

ISO-ENZYME ELECTROPHORESIS: METHODS

Vertical poly-acryl amide gel electrophoresis (PAGE)

SANDWICH ASSEMBLING:

1. Wash glass plates with detergent and rinse with distilled water.
2. Wipe plates with 70% ethanol.
3. Assemble plates and make sure the spacers (1mm) are perfectly aligned with the glass plates.
4. Use grey clamps to hold plates in position.
5. Fit the sandwich into the gel caster stand.
6. Level the caster and insert black clamps with notch facing upwards.
7. Turn black clamps, but not more than 180°.

POURING OF THE GEL:

Make a 6% ACRYL AMIDE: BIS-ACRYL AMIDE SOLUTION (33:1):

1. 18ml of the 40% stock solution (acryl:bisacryl 33:1) kept in fridge.
2. 3ml 10x TBE (Tris-borate EDTA) buffer (0.025m final concentration) pH 8.5.
3. Add distilled water to get a final volume of 120ml.

THIS SOLUTION IS ENOUGH FOR 4 GELS.

4. Just before pouring the gels take 20ml out of the gel solution and add 200ul refrigerated 10% APS (Ammonium persulphate) and 20ul TEMED (N, N, N^9, N^9 – Tetramethylethylenedianine). Then pour the base gel of all 4 gels to seal the bottom.
5. Wait 10-15 min. for polymerization of the base gel.
6. To the remaining gel solution add 600 ul 10% APS and 60ul TEMED. Make sure it is mixed thoroughly before pouring it on top of the polymerised base gel layer.
7. Insert the 1mm combs, making sure no air is trapped underneath the combs and remove all the air bubbles.
8. Leave gel for 1hr to polymerize completely, before removing the combs.

PRE-RUNNING OF GEL

1. After gel polymerization (1hr), take out the combs and wash out the wells carefully with distilled water.
2. Add 1 x TBE buffer to the wells and put top buffer chamber on top of the gels.
3. P[illegible]k clamps and seal chamber to gel plates to prevent leaking of buffer.
4. Add a small quantity of buffer to the top buffer chamber to see if there are any leaks in the set up.
5. Put top buffer chamber into lower buffer chamber (containing pre-cooled 1 x TBE buffer 0.1M pH 8.5) with the electrodes next to each other.
6. Fill top buffer chamber to the level indicated on the buffer chamber.

7. Put the safety lid on and pre-run the gel for 1hr at 300V (with no regard to milliamps). Pre-running cleans the gel of other ions present and the gel is now ready for loading of the samples.

SAMPLE PREPARATION AND LOADING

Mosquito samples are homogenised separately in 1.5ml eppendorf tubes using 15ul grinding buffer, and an homogenising stick.

1. Use liquid nitrogen to squash each mosquito in the 1.5ml eppendorf tube.
2. Add 15ul grinding buffer and squash sample further.
3. Clean the homogenising stick against the side of the eppendorf tube.
4. All specimens are left on ice while crushing/grinding is being carried out.
5. Spin homogenised specimens in a microcentrifuge at 14 000 rpm for 3 minutes. Make sure the eppendorf hinges are on the outside and that the centrifuge is balanced.
6. After the pre-run phase, the gels can be taken out of the electrophoresis unit. Save the buffer from the top buffer chamber making sure that the positive electrode is kept dry while the buffer is being poured off.
7. Use the micro-syringe and load 3ul of blood in the first well on each plate as a marker.
8. The micro-syringe can be rinsed in between samples using distilled water. If the plunger is sticky in the barrel, take the plunger out, rinse and wipe clean, and place back in barrel.

9. Loading of specimens depends on the enzyme system being tested.

    *load 1ul/15ul for AAT, EST and XDH

    *Load 3ul/15ul for ODH and MPI

10. Top buffer chamber can be put back onto the gels as described for the pre-run.

RUNNING OF PAGE GELS

1. Gels are run for 10 minutes at 100V (The milliamps should never exceed 40).
2. After 10 minutes the volts are turned up to 250V (watch mA) till the blood has travelled for 2cm. (XDH gels will rung up to 4-5 cm for a good separation).
3. The gels are rung for a further 10 minutes at 100V.
4. Gels can now be removed and stained for the relevant enzyme systems.

Colonies utilised in standardising the PAGE electrophoretic system for use in our study are existing colonies of the *An. gambiae* complex housed and maintained at the Botha De Meillon Insectary, SAIMR, Johannesburg.

SPECIES	COLONY NAME	ORIGIN
ARABIENSIS	KGB	Kanyemba, Zimbabwe
	Malmix	Malahlapanga, Kruger Park, South Africa
	Ma	Maputo, Mozambique
	Man	Mananga, Swaziland
	Dakar	Dakar, Senegal
GAMBIAE	Ian P20	Iworo, Nigeria
	16 CSS	Lagos, Nigeria
	GAG	Georgetown, The Gambia
MERUS	Mafayeni	Mafayeni, Natal, South Africa
	Merdar	Dar-es-Salaam, Tanzania
QUADRIANNULATUS	Skuquad	Skukuza, Kruger Park, South Africa

ISO-ENZYME ELECTROPHORESIS: RECIPES; STAINING TECHNIQUES

recipes for stains, buffers, grinding solution and gels

40% Stock Acryl:Bisacryl (33:1)

120g acrylamide

3.6g bisacrylamide

Made up to a final volume of 300 ml in WARM distilled water swirling GENTLY by hand. (DO NOT use stirrer).

6% (33:1) Gels From 40% Stock Solution

Quantities used to make 4 gels

18ml 40% stock acryl:bisacryl (33:1)

3ml 10 x TBE buffer

99ml distilled water

120ml Total volume

To the 120ml mixture add 600ul APS 10% (stored in refrigerator) followed by 60ul TEMED. Pour gels immediately after adding TEMED.

10% APS must be made fresh each week, and stored at 4°C.

10 x TBE (1m) Buffer Stock Solution (pH 8.9)

98.25 g	TRIS (MWt = 121.1)
17.50g	BORIC ACID (MWt = 62)
5.56g	EDTA tetrasodium (MWt = 380.2)

made up to a final volume of 1 litre.

1 x TBE (0.1 M) Running Buffer

600ml of 10 x TBE made up to 6 litres in distilled water.

Running buffer may be used for 3 runs only.

40 x TC Conc. Buffer (Tris - Citrate, pH 7.1)

91.7g	TRIS (MWt 121.1)
47.0g	CITRIC ACID (MWt 210)

Made up to 1 litre in distilled water.

Grinding Solution

10%	SUCROSE
1%	TRITON X 100 DETERGENT
1:3	DILUTION OF TRIS-CITRATE TANK BUFFER
	BROMOPHENOL BLUE (Add enough for good colour).

Octanol Dehyrogenase (ODH) And Superoxide Dismutase (SOD) (Mahon *et al.*, 1976)

10.0mg	NBT (Nitro-blue tetrazolium)
12.5mg	NAD (Nicotinamide adenine dinucleotide)
2.0mg	PMS (Phenazine methosulphate)
0.1ml	Octanol
0.5ml	Ethanol
50.0ml	0.05m Tris-HCl buffer

Procedure

1. Add NAD, NBT, Octanol, ethanol and Tris-HCl in a beaker. Stir using magnetic stirrer.

2. Add PMS stirring until a pinkish purple colour appears.

3. Pour over gel and place in dark oven (37°C) until blue bands show up (ODH).

4. Add extra PMS and put back in oven until SOD bands show up.

5. Rinse gel and fix with 1% acetic acid.

Aspartate Aminotransferase (AAT) (Munstermann, pers.comm., unpublished laboratory notes)

50mg	Cysteine sulphonic acid
50mg	α Ketoglutaric acid
32 units	(0.027 ml) Glutamate dehydrogenase
20mg	NAD
20mg	NBT
50ml	1M Tris - HCl pH 8.0

Procedure

1. Mix all ingredients in a beaker using a stirrer.

2. Pour over gel and incubate at 37°C adding a touch of PMS after 15 minutes.

3. Rinse and fix with 1% acetic acid once bands show up.

Esterase (EST) (Steiner and Joslyn 1979)

0.66g	Sodium phosphate (monobasic)
0.27g	Sodium phosphate (dibasic)
50ml	distilled water
4ml	1% α - naphthyl acetate in acetone (made up fresh every week)

1. Add all ingredients into a beaker and place on a magnetic stirrer

2. Pour half over gel. Place gel and leftover stain in dark cupboard for 15 minutes

3. Use left over stain to make 50mg Fast blue RR into a paste. Pour this over gel.

4. Leave at room temperature in the dark until bands show up. Rinse and fix gel with 1% acetic acid.

Xanthine Dehydrogenase (XDH) (modified Miles 1978)

20mg	NAD
12mg	NBT
1mg	PMS
20mg	hypoxanthine
4 drops	10% Potassium hydroxide
50ml	0.05 Tris - HCl pH 7.1

Procedure

1. Add all ingredients and stir in beaker in the DARK until hypoxanthine dissolves (10 - 20 minutes).

2. Pour over gel and incubate in oven (37°C) until bands show up.

3. Rinse and fix with 1% acetic acid.

Mannosephosphate Isomerase (MPI) And Glycerol Dehydrogenase (GCD) (modified Harris and Hopkinson 1976)

14mg	NADP (activity declines after some time)

7mg	NBT
14mg	mannose - 6 - phosphate
28mg	hydrated magnesium chloride
8mg	Glucose phosphate isomerase
30ul	Glucose - 6 - phosphate dehydrogenase (activity declines once opened)
30ml	0.2m Tris - HCl (pH 7.5)
5ml	Glycerol

Procedure

1. Add all ingredients and stir in beaker for at least 10 minutes.

2. Pour over gel and incubate at 37°C. After 30 minutes add a little extra PMS and incubate until bands show up.

3. Rinse and fix gel with 1% acetic acid.

APPENDIX II

PCR PROTOCOL

1. Legs from each alcohol preserved sample were dried briefly on a paper towel, placed in a microcentrifuge tube (0.5ml) and kept on ice throughout the procedure.
2. 12.5ul aliquots of a PCR mastermix were placed into the microcentrifuge tubes with the tissue sample and the tip of the adjustable Gilson pipette was used to homogenise the tissue.

   Mastermix consisted of 0.5 units of taq polymerase (Advanced Biotechnologies Thermostable Taq Polymerase), 1.25ul 10 x PCR buffer, 125ul of each nucleotide, 1mM $MgCl_2$ and 0.3uM of each of the 5 primer (sequences as per Scott *et al.*, (1993), produced by the Biochemistry Department of the University of Cape Town).
3. Autoclaved distilled water was added to bring the total volume to 12.5ul.
4. The reaction mixture in each tube was overlaid with mineral oil (35ul).
5. Each tube was placed in a Hybaid thermal cycler and run through a 30 cycle reaction which consisted of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72° for 30 seconds. An autoextension of 10 mins at 72°C completed the procedure.
6. 10ul of the amplified product of each sample was mixed with 4ul of a ficoll loading dye and loaded onto a prepared 2.5% agarose gel stained with ethidium

bromide (0.3ug/ml gel). A 1 kilobase DNA ladder was also loaded to help measure sizes of unknown fragments.

7. The samples were run electrophoretically in a TBE buffer at 100 milliamps until the bromophenol blue had migrated ± 3cm.
8. The DNA fragments were visualied on an ultrviolet transilluminator and photographed on polaroid film.

APPENDIX III

TWO PAPERS SUBMITTED FOR PUBLICATION TO THE *JOURNAL OF THE AMERICAN MOSQUITO CONTROL ASSOCIATION.* REVISED AS PER EDITORIAL REQUEST, ACCEPTED FOR PUBLICATION JANUARY 1998.

Attached is a copy of the letter of acceptance from the Editor of the *Journal of the American Mosquito Control Association* with recommended revision of the original submitted manuscript. These revisions have been made and the revised two manuscripts are included here in their entirety despite duplications that occur, eg. with tables, in the body of the thesis. The manuscripts have not been given thesis page numbers but are included rather as an insert into the thesis.

All references to the name proposed for the new species have been deleted or replaced with "xxx" since there is no desire to infringe the *International Code for Zoological Nomenclature* and comprise the name before it appears in a valid publication. Throughout the thesis the new species is referred to as "*An. listeri sp. B*".

The contributions of each of the three authors to the papers are as follows: R.A. Khan collected the specimens, carried out comparative analyses (morphological and cytogenetic) of the new species with *An. listeri* and drafted and revised the manuscripts. M. Coetzee co-supervised the work, helped with the E.M. photographs of eggs and the analysis of the data, and gave critical comment on the manuscript. R.H. Hunt, supervisor, assisted with the field collections, generated the chromosome maps and gave critical comment on the manuscript.

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December 16, 1997

Dr. R. Abdulla-Khan
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Dear Dr. R. Abdulla-Khan,

I have now received two reviews of manuscript M4598: "A description of *Anopheles (Cellia)* sp. nov. (Diptera, Culicidae) from Kasane, Botswana" by yourself, M. Coetzee and R.H. Hunt. Both reviewers were interested in and recognized the importance of your finding a new species in the *Anopheles* subgenus *Cellia*. However, both felt strongly that you have not followed the proper format in describing a new species and that your manuscript has an inappropriate emphasis on the habitat and biology of the species, rather than a formal taxonomic description. I have read through your manuscript as well and think that you have done an impressive amount of work but have to agree with the reviewers. I personally think that you have two manuscripts here and propose that you submit two new manuscripts.

In the first manuscript, you need to follow both reviewers' recommendations and write a formal taxonomic description of *Anopheles (Cellia)* sp. nov. This should follow The International Code of Zoological Nomenclature and include telegraphic descriptions and drawings of larval and pupal stages, tables of setal counts as well as corrected drawing of wings. Follow the morphological terminology of Harbach and Knight (1980). Please use some of the recent species descriptions in JAMCA as examples of the correct format. The most recent in JAMCA is:
Ribeiro, H. 1997. New species of *Toxorhynchites* (Diptera : Culicidae) from Macau (China). JAMCA 13: 213-217.
Also, if possible you should discuss proper descriptions of new species with Dr. Jupp at the National Institute for Virology. He is an excellent mosquito taxonomist in South Africa. The second manuscript would constitute a description of the collection methods, larval habitats and other observations that you have made for *Anopheles*

If this is agreeable to you, please make the corrections recommended by both reviewers and divide the material into two manuscripts, each containing the information outlined above. After you have made these changes return the manuscripts to me on disk. If possible provide the manuscripts in a Word 6.0, Wordperfect 5.1 or 6.0 format. I will edit the manuscript and, if necessary return it to you for further consideration. Then we will forward it to the JAMCA office for final processing. I will tell the editorial office to publish these as companion papers in a single issue. In that way you can cross reference material in the two papers in your revisions.

Best regards,

William C. Black IV
Co-Editor
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DESCRIPTION OF *ANOPHELES (CELLIA) Xxxxxxx* SP.NOV. (DIPTERA, CULICIDAE) FROM KASANE, BOTSWANA.

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ABSTRACT

Anopheles (Cellia) xxxxxxx, a new mosquito species from Kasane, northern Botswana is described on the basis of the examination of a type series of 37 females and 18 males. Diagnostic features on the egg, larva and pupa are presented. Comparisons of the new species with close relatives, *An. listeri*, and *An. azevedoi* are made.

Running title: Description of *An. xxxxxxx* **sp.nov.**

INTRODUCTION

During a mosquito survey carried out in 1995 in Kasane, Chobe District, north-eastern Botswana (Abdulla-Khan et al. 1998), specimens of an anopheline mosquito belonging to a new, undescribed species were collected biting humans indoors and feeding on cattle in enclosures.

Wild females were returned to the laboratory, isolated for ovipositing, and the progeny reared to adults as individual families (Hunt and Coetzee 1986). Each pinned adult had associated larval and pupal pelts mounted on slides. Eggs were stored in 70% alcohol for scanning electron micrography.

The descriptive terminology used is that of Harbach and Knight (1980). Diagnostic characters for separation of *Anopheles listeri* De Meillon, *An. azevedoi* Ribeiro and the new species are given.

TAXONOMIC TREATMENT

***Anopheles (Cellia) xxxxxxxx*, new species** (Figs. 1-4, Tables 1,2).

Female: Wing length 3.16-3.60 mm. Wing (Fig. 1a): largely pale, with pale and dark areas well-contrasted. Median dark (MD) areas of costa and subcosta separate, approximately equal in length. Apex of vein R_3 with dark scales extending to the fringe spot. Palps: identical to *An. listeri* De Meillon. Legs: femora dark: hind tibiae narrowly pale at base and apex, fore and mid-tibiae pale at apex only; all tarsomeres dark.

Male: Wings and legs as in female. Genitalia: as in *An. listeri* (De Meillon 1931, Gillies and

De Meillon 1968). Palps (Fig. 1b,c): of two types, one identical to *An. listeri* (Fig. 1b) and the other with a paler overall appearance (Fig. 1c); shaft dark proximally, followed by a long pale area; club widely pale at apex followed by a narrow dark region preceding a wide pale area medianly; pale base of club contiguous with pale area on shaft.

Pupa: Full setal counts are provided in Table 1. The following are diagnostic for *An. xxxxxxx*: *Abdomen*: seta 1-II, 7-8 branches; 1-III, 6-7 branches; 1-VII, simple, same length as segment (Fig. 2); 5-III to 5-VII with 6-7, 4-6, 4-5, 3-4 and 2-3 branches respectively; 9-VI to 9-VII simple, equal to or less than half the segment length.

Larva: Full setal counts are provided in Table 2. The following are diagnostic for *An. xxxxxxx*: *Prothorax:* setae P_1 and P_2 branched, on small separate tubercles (Fig. 3a); P_1 9-10 branches, P_2 8-10 branches. *Abdomen:* 1-I and 1-II rudimentary, leaflets lanceolate and undifferentiated with 16 branches (Fig. 3b); 1-III to 1-VII fully developed with 16 moderately broad leaflets, well-defined shoulders and short, blunt tipped filaments varying in length (Fig. 3c).

Egg (Fig. 4a): shiny, silver-grey, boat-shaped egg with broad dorsal surface (deck). The undivided deck is surrounded by a well-developed, narrow, striated frill, interrupted in the middle on each side by well-developed floats consisting of 16 float chambers. Under light microscopy, two dark, irregular patches occur across the deck on each side of the mid-centre of the deck. This coloration is not apparent on scanning electron micrographs (Fig. 4a). The exochorion in these patches consists of relatively broad, irregular bosses when compared with the remainder of the upper surface of the deck which comprises finer, smaller bosses. Numerous, large, lobed tubercles are located at both anterior and posterior ends of the deck.

The micropylar apparatus is small covered by a thin, unornamented layer of exochorion. The micropylar channel is flat, with a cone-shaped micropylar process. The base of the micropylar apparatus is smooth. The undersurface has a fine, reticular pattern.

Type data. *Holotype*. Female: Kasane, Botswana (17°48'S, 25°09'E), February 1995. No. BOT 169.10. Associated larval and pupal pelts mounted on slide with same data and identification number. *Paratypes*: 5 females (BOT 169.1, 2, 3, 6 and 7) and 4 males (BOT 169.4, 5, 8 and 9) with associated immature pelts with same data and identification number. All specimens reared from eggs obtained from a single wild female, collected in a cattle enclosure by R. Hunt and R.A. Khan. Deposited in the collection of the South African Institute for Medical Research (SAIM). Eggs of type family subjected to electron microscopy and photographed (Fig. 4a).

Material examined: In addition to the type material, other specimens examined were progeny from 10 wild females (BOT 116, 135, 147, 148, 166, 168, 170, 171, 172, 195) collected from either a cattle enclosure (22 females, 9 males) or indoors biting humans (9 females, 5 males), February 1995. Deposited in the SAIM.

Etymology: The new species is named in honor of Sir Seretse Khama, the late president of Botswana, 1966-1980.

DISCUSSION

Anopheles xxxxxxx **sp.nov.** belongs to the *An. listeri* group sharing most morphological features in the adult, pupal and larval stages. Notable differences between *An.*

xxxxxxxx, *An. listeri* and *An. azevedoi* (Ribeiro 1969), a third member of the group, are presented in Table 3. Adults of all three key out to section VII no. 12 of the key to female *Anopheles* in Gillies and Coetzee (1987). Larvae key out to section IX of the larval key but are easily separated by the characters in Table 3. The eggs of *An. xxxxxxxx* with floats (Fig. 4a) differ from *An. azevedoi* and *An. listeri* which have no floats (Fig. 4b and Gillies and Coetzee, 1987).

The bionomics and cytogenetics characteristics are given in Abdulla-Khan et al. (1998).

ACKNOWLEDGMENTS

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Table 1. Full setal counts for pupal specimens of *An. xxxxxxx* sp. nov. (n = 54).

SETA	RANGE	SETA	RANGE	SETA	RANGE
MET		ABD III		ABD VI	
10	1-4	11	Simple	5	3-4
11	2-4	14	Simple	6	2-3
12	1-3	ABD IV		7	1-4
ABD I		0	Simple	8	1-3
1	Fan	1	Simple	9	Simple
2	4-6	2	4-5	10	Simple
3	2	3	3-7	11	Simple
4	5-6	4	1-3	14	Simple
5	1-3	5	4-6	ABD VII	
6	Simple	6	1-3	0	Simple
7	7-10	7	4	1	Simple
9	Simple	8	3-4	2	3-4
ABD II		9	Simple	3	1-2
0	Simple	10	Simple	4	Simple
1	7-8	11	Simple	5	2-3
2	6-8	14	Simple	6	1-4
3	2-3	ABD V		7	2-3
4	4	0	Simple	8	Simple
5	2-3	1	Simple	9	Simple
6	Simple	2	5-6	10	1-3
7	3-6	3	1-3	11	1-2
8	2	4	1-2	14	Simple
9	Simple	5	4-5	ABD VIII	
10	1-2	6	3-4	0	Simple
ABD III		7	2-4	4	Simple
0	Simple	8	3-5	9	3-8
1	6-7	9	Simple	14	Simple
2	5-8	10	Simple	1	Simple
3	1-3	11	Simple	Paddle	
4	3-4	14	Simple	1	Simple
5	6-7	ABD VI		2	1-2
6	3-4	0	Simple		
7	2	1	Simple		
8	2-4	2	5-6		
9	Simple	3	Simple		
10	1-3	4	1-2		

Table 2. Full setal counts for larval specimens of *An. xxxxxxx* sp. nov (n= 42).

SETA	RANGE	SETA	RANGE	SETA	RANGE	SETA	RANGE
Cranium		12	5-10	6	Simple	13	1-2
0	Simple	13	3-4	8	2-3	-VIII	
1	Simple	Abd-I		9	Simple	0	Simple
2	Simple	1	16	10	2	1	Simple
3	Simple	2	Simple	11	Simple	2	3-6
4	Simple	3	Simple	12	Simple	3	Simple
5	7-9	4	3-4	13	1-2	4	Simple
6	3	5	1-3	Abd-V		5	1-2
7	3-4	6	3-6	0	Simple		
8	5-8	7	4-9	1	16		
9	9-12	8	Simple	2	1-2		
10	14-17	9	Simple	3	2-4		
11	14-18	10	1-2	4	Simple		
12	1-3	11	Simple	5	Simple		
13	3-5	12	Simple	6	1-2		
14	3-4	13	2-4	7	3-8		
Antenna		Abd-II		8	2-3		
2	Simple	1	16	9	Simple		
3	Simple	2	Simple	10	2		
4	3-5	3	3	11	1-2		
Prothorax		4	Simple	12	Simple		
1	9-10	5	2-3	13	1-3		
2	8-10	6	4-7	Abd-VI			
3	Simple	7	4-9	0	Simple		
8	2-3	8	2-3	1	16		
14	2	9	2-4	2	1-2		
Mesothorax		10	1-2	3	Simple		
1	2-3	11	Simple	4	Simple		
2	Simple	12	Simple	5	Simple		
3	Simple	13	1-2	6	1-2		
4	Simple	Abd-III		7	3-8		
5	Simple	0	Simple	8	1-2		
6	Simple	1	16	9	Simple		
7	Simple	2	1-2	10	1-2		
8	2-3	3	2-4	11	Simple		
9	Simple	4	2-4	12	3-4		
10	2-4	5	Simple	13	1-3		
13	4-5	6	3-5	-VII			

14	5-6	8	1-3	0	Simple		
Metathorax		9	Simple	1	16		
1	Simple	10	2	2	1-2		
2	Simple	11	1-2	3	Simple		
3	Simple	12	Simple	4	Simple		
4	Simple	13	1-3	5	Simple		
5	5-8	Abd-IV		6	1-2		
6	2-4	0	Simple	7	3-8		
7	5-8	1	16	8	1-2		
8	4-9	2	1-2	9	Simple		
9	5-11	3	2-4	10	1-2		
10	4-8	4	2-4	11	1-2		
11	4-9	5	Simple	12	2		

Table 3. Characters used to distinguish *An. xxxxxxxx* sp.nov.. *An. listeri* and *An. azevedoi.*

CHARACTER	*An. xxxxxxxx*	*An. listeri*	*An. azevedoi*
Adult	None	None	None
Pupae			
Seta 1-VII	Simple, as long as segment VIII	1.5 length of segment VIII	0.6 length of segment VIII
Seta 9-VII	≤0.5 length of segment VIII	≥0.5 length of segment VIII	Equal to length of segment VIII
Larvae			
Mesothorax seta M-9	Simple	Simple	Branched (De Meillon + Van Eeden 1976). Simple (Ribeiro 1969)
Egg	With floats	No floats	No floats

FIGURE LEGENDS

FIG. 1. a. Female wing (from right to left): C (costa); Sc (subcosta); BD (basal dark); HP (humeral pale); HD (humeral dark); PSP (presector pale); PSD (presector dark); SP (sector pale); ASP (accessory sector pale); MD (median dark); SCP (subcostal pale); PD (preapical dark); PP (preapical pale); AD (apical dark); AP (apical pale); PFS (pale fringe spot). Veins (R_1, R_2, R_3, R_4, R_5).

b. *An. listeri* male palp (also seen in some *An. xxxxxxx* specimens)

c. Male palp seen in *An. xxxxxxx* only.

FIG. 2. Pupa of *An. xxxxxxx* : seta 1-VII equal in length to abdominal segment VIII.

FIG. 3. Larva of *An. xxxxxxx:*

a. Prothoracic setae 1-2

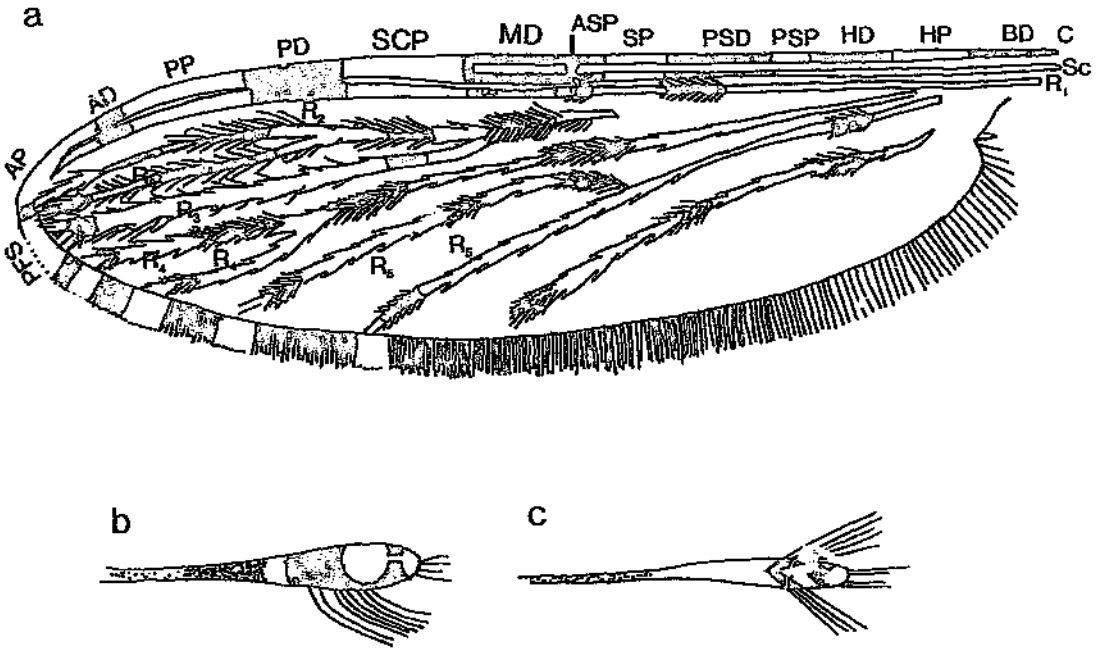
b. Abdominal palmate setae 1-II underdeveloped

c. Fully developed palmate seta 1 on segments III-VII.

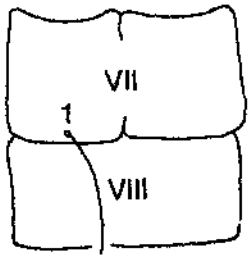
FIG. 4. a. Egg with floats – *An. xxxxxxx*

b. Egg without floats – *An. listeri*

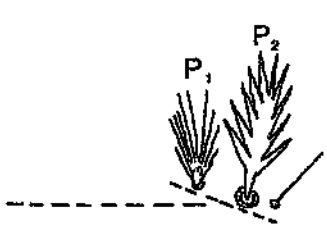
Fr (frill); De (deck); FR (float ridge); F (float)


a
PP
PD
SCP
MD
ASP
SP
PSD
PSP
HD
HP
BD
C
Sc
R1
AD
AP
R2
R3
R4
R5
PSP
b
c

Pupa



Larva

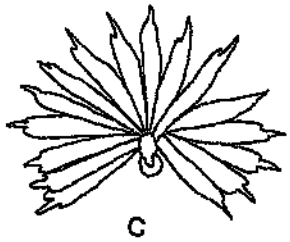


a

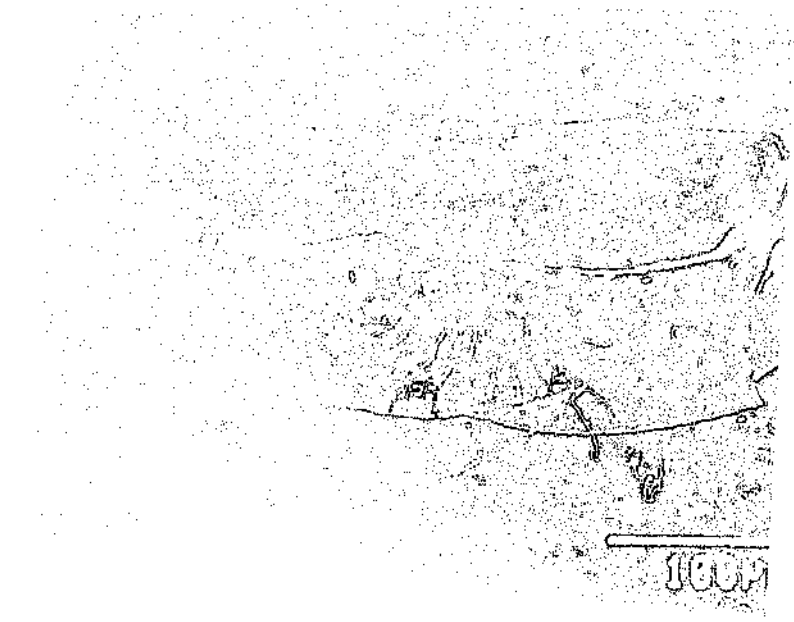
1–II

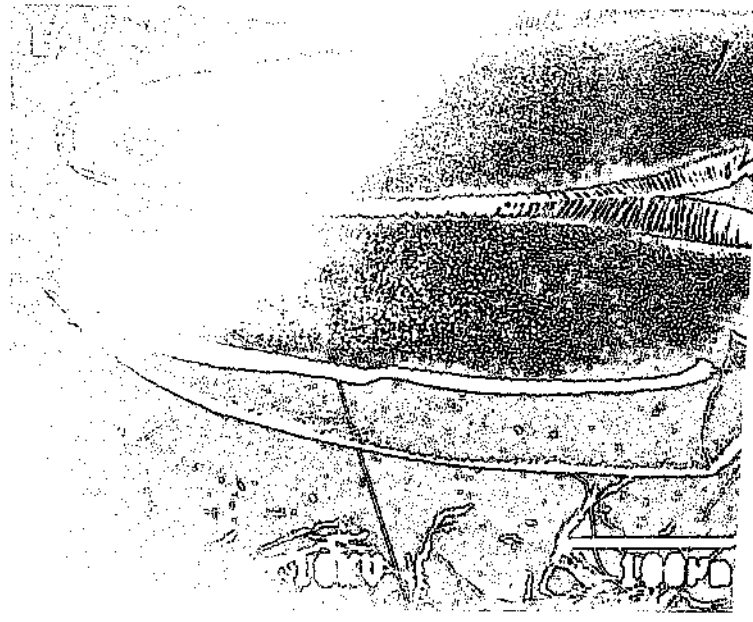
b

1–III – 1–VII



c





ABSTRACT

Morphological analysis of anopheline mosquitoes from Kasane, Botswana, revealed a new species, *Anopheles xxxxxxx*, closely related to *azevedoi* and *listeri*. A description of the type locality and biological characteristics of *An. xxxxxxx* are given. Comparisons are made with *An. listeri* and *An. azevedoi*. The banding patterns of the giant polytene chromosomes of *An. xxxxxxx* were compared with those of *An. listeri* and found to be homosequential.

Running title: Biology and cytogenetics of *An. xxxxxxx*

INTRODUCTION

A recent survey of the mosquitoes from Kasane, Botswana revealed an new species *Anopheles xxxxxxxx* Abdulla-Khan, Coetzee and Hunt (1998), that is morphologically very similar to *An. listeri* De Meillon and *An. azevedoi* Ribeiro. The present paper summarises our knowledge of the distribution and biological characteristics of the three species and presents a photomap of the polytene chromosomes of *An. xxxxxxxx*.

DISTRIBUTION

Anopheles listeri is widespread in southern Africa occurring in Zimbabwe, South Africa, Namibia and southern Angola (fig. 57 in Gillies and De Meillon 1968). It was collected in Botswana at Mowana Lodge, Chobe, in the cattle enclosure and pig pen (Fig. 1).

Gillies and Coetzee (1987 (fig. 28)) provide a map of the distribution of *An. azevedoi* showing several records along the arid coastal belt of Angola (Ribeiro 1959, 1974, Ribeiro and Ramos 1975) and one from the Uppington district of the Northern Cape Province, South Africa (De Meillon and Van Eeden 1976).

An. xxxxxxxx is known only from the type locality, Kasane, Chobe District, north-eastern Botswana. Collections were carried out at four sites: Chobe safari Lodge (17°48'S, 25° 09'E), Mowana Lodge (17° 48'S, 25° 11'E), a cattle enclosure and a pig pen on the Kazangula road (17° 48'S, 25° 19'E) (Fig. 1). *An. xxxxxxxx* was collected only at the Mowana Lodge and the cattle enclosure. The area is in close proximity to the Zambezi and Chobe rivers and includes two saline hot springs on the Chobe river side whose activity depends on the rainfall in the region. The mean altitude for Kasane is 935 m above sea level and the min–max temperature range

during the rainy season is 19-30°C. *An. xxxxxxxx* has not been collected from any other area.

ADULT BIOLOGY

Little is known about the adult biology of *An. listeri* other than that in Namibia, the adults frequ 'y occur in houses and readily bite humans (Gillies and De Meillon 1968). De Meillon (1931) thought that *An. listeri* might be implicated in epidemic malaria in Namibia, despite the negative results of a small number of dissections for malaria parasites.

In Angola, adults of *An. azevedoi* were captured resting in house, outdoors, particularly in crevices in the ground, in rocky cliffs and also in vegetation. Females were also collected biting humans outdoors (Ribeiro and Ramos 1975). Salivary gland dissections for sporozoites were carried out on 109 females with negative results (Ribeiro and Ramos 1975).

Adult females of *An. xxxxxxxx* were collected biting humans indoors (Mowana Lodge) and feeding on cattle in enclosures (Kazangula road).

LARVAL BIOLOGY

Anopheles listeri shows high tolerance to salinity, with the larvae being found in saline water that is > 1.5 times (52.5g/l, NaCl) the concentration of sea water (Ribeiro and Ramos 1975). Gillies and De Meillon (1968) however, state that the larvae are found in open sunlit pools and do not mention tolerance to salt water.

Anopheles azevedoi occurs frequently in highly saline pools along the south-western coast of Angola. Larval habitats range from tidal pools, saline canals and saltpans to brackish wells (Ribeiro 1974, Gillies and Coetzee 1987). The salinity of the most common larval breeding sites

approaches that of sea water (35-36g/l, NaCl) however, some larvae have been found in water with salinity of up to 400% sea water. In conditions of low salinity, *An. azevedoi* occurs in association with *An. listeri* (Ribeiro 1974, Ribeiro and Ramos 1975). In the upper Karoo, Northern Cape Province, the larvae of *An. azevedoi* were found in saline pools (14.1g/l, NaCl) in association with fairly large numbers of *An. listeri* (De Meillon and Van Eeden 1976).

The larval habitat of *An. xxxxxxxx* is unknown, but is probably in saline hot springs. An X-ray diffraction analysis of soil samples taken from the hot springs around Kasane showed the presence of Halite (NaCl). In the laboratory, egg batches obtained from wild females were divided 50:50 into bowls containing either distilled water or 25% seawater equivalent (8 g/l NaCl). All first instar larvae died in the distilled water and survived to adulthood in 25% seawater, indicating that *An. xxxxxxxx* is an obligatory salt-water breeder.

CYTOGENETICS

Giant polytene chromosomes are found in ovarian nurse cells of half-gravid anopheline females (Coluzzi 1968). The banding patterns on these chromosomes have, in many cases, provided a taxonomic tool for the identification of morphologically similar species, for example, the *Anopheles gambiae* Giles complex (Coluzzi et al. 1979), the *An. marshallii* (Theobald) complex (Lambert 1979).

The F_1 female progeny of the *An. xxxxxxxx* that were not preserved for museum specimens, were held in cages for five days before being offered a blood meal. Those that became half-gravid were dissected and the ovaries stored in Carnoy's preservative (Hunt and Coetzee 1986). Chromosome preparations were made according to the method of Green and Hunt (1980).

Due to some difficulty in stimulating ovarian development in unmated females, only five ovaries were obtained from *An. xxxxxxx*. Of these, only two preparations had chromosomes that could be scored. These were compared with the chromosomes of *An. listeri* (Fig. 2) from Sautini, Northern Province, South Africa. No differences were seen in the banding patterns of the two species. A similar situation exists in the *An. gambiae* complex where *An. quadriannulatus* (Theobald) species A and B, share homosequential banding sequences (Hunt et al. 1998).

The polytene chromosomes of *An. azevedoi* are unknown.

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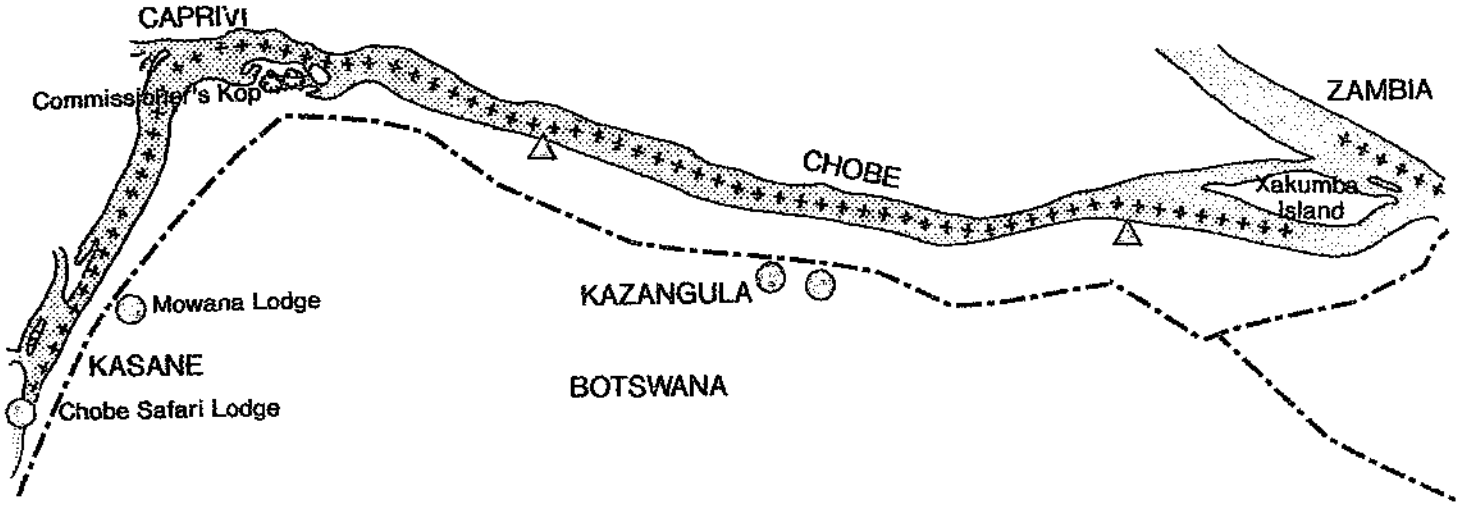
FIGURE LEGENDS

Fig. 1. Map of the Chobe district, north-eastern Botswana, showing four collection points (⊙) and the localities of two hot springs (△).

Fig. 2. The polytene chromosome complement of *Anopheles listeri* from Sautini, Northern Province, South Africa. C = centromere.


CAPRIVI
Commissioner's Kop
ZAMBIA
CHOBE
Xakumba Island
Mowana Lodge
KAZANGULA
KASANE
Chobe Safari Lodge
BOTSWANA

X
C
2
3
4
5

APPENDIX IV

SPECIES CONCEPTS

The Isolation Concept of species

Dobzhansky (1937) believed that the species gene pool was delimited through the action of "isolating mechanisms". He initiated a line of thought that was to influence the majority of biologists working in the field of species concepts and modes of speciation long after he proposed that two distinct populations are reproductively separated by *ad hoc* characteristics which he termed "isolating mechanisms".

Isolating mechanisms fall into two distinct categories: (i) Premating isolating mechanisms (seasonal and habitat isolation; ethological isolation; mechanical isolation) and (ii) Postmating isolating mechanisms (gamete mortality; zygote mortality; hybrid inviability; hybrid sterility). These criteria emphasise events that take place when two species meet, and put little emphasis on how different sexes within the same species behave when they come into contact with one another (Paterson 1985) even though conspecific interactions are of greater importance to the reproductive success of a species.

In the Isolation Concept the selection of isolating mechanisms is brought about at the interface of two populations that have diverged. It is suggested that two populations in allopatry diverge genetically in such a way that even though no ethological barriers are faced between the two entities, any resulting hybrids are disadvantaged (sterile, not viable etc.).

Natural selection then favours those individuals that mate with their "own kind" and any incipient isolating mechanisms are therefore reinforced (Ayala *et al.*, 1974). This speciation by reinforcement requires selection to take place in sympatry which is unlikely because the smaller population of disadvantaged hybrids would become extinct as a result of negative heterosis (Paterson 1978).

The Recognition Concept of species

Paterson (1978) using evidence quoted by several authors in support of the isolation theory, proceeded to demonstrate some inherent flaws in both their experimental and their theoretical arguments. He went on to redefine a species as that most inclusive population of individual, biparental organisms which share a common fertilisation system (Paterson, 1985). He suggested that individuals in their normal habitat share a common specific mate recognition system (visual, auditory, chemical, tactile and/or other) which is an essential prerequisite to fertilisation of a conspecific mate. The Recognition Concept does not depend on the relationship of one species to populations of other species. In allopatry, if the specific mate recognition system changes to such an extent that members of the original population are no longer recognised as potential mates, speciation can be said to have occurred. If the populations return to sympatry without significant changes to the recognition system and fertilization does occur, one of two possibilities may arise. If the hybrid offspring are sterile, the smaller population will go to extinction. If, on the other hand, the hybrids are only partly infertile, natural selection will eliminate the cause of the infertility. In large, randomly

mating populations, any genetic changes accumulated in allopatry would become polymorphisms within a homogeneous population.

The Recognition Concept of species considers species to be the incidental consequences of adaption to a new environment. This concept is a non-relational one in contrast to the Isolation Concept of species.

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