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Coetzee, Maureen

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A MORPHOMETRIC STUDY OF FOUR MEMBERS OF THE ANOPHELES (CELLIA) GAMBIAE COMPLEX (DIPTERA:CULICIDAE)

by Maureen Coetzee

Thesis submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg,
in fulfilment of the requirements for the
degree of Doctor of Philosophy.

JOHANNESBURG.
October 1986.

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.

M. Coetzee

ABSTRACT

The Anopheles gambiae group of mosquitoes contains species which are considered to be the most efficient vectors of human malarial parasites in Africa. All the species in the group have been defined genetically and the most accurate current method of identification is chromosomal. The ease with which a field entomologist can identify vectors has a direct bearing on the methods and effectiveness of control programmes and epidemiological studies. Classical taxonomy using morphology to identify anophelines was the most convenient method available. However, the members of the An. gambiae group are virtually identical in their external appearance. A concerted attempt has been made here to find some simple morphological characters which may be used to identify the members of the group found in southern Africa.

Wild-caught females, their F-1 progeny, and wild larvae from numerous localities in southern Africa were used in this study. The identification of the species was either chromosomal, electrophoretic or both. A method is provided for the correlation of mounted museum specimens with photographs of chromosomes and electromorphs.

The results of the morphological study on the adult females show that hind leg banding patterns can be used to group the major vectors gambiae/arabiensis and the lesser or non-vectors merus/quadriannulatus. No structural characters were found which separate more than 75% of individual An. gambiae and An. arabiensis in a simple way. Using the palp ratio and coeloconic sensilla number, An. quadriannulatus and An. merus could be effectively separated. Characters on the immature stages can be used to identify An. merus but not to separate the three freshwater breeding members An. gambiae, An. arabiensis and An. quadriannulatus. Finally, a computer multivariate discriminant function analysis of the morphological characters studied separated 97% of the individuals used.

Classical taxonomy for the identification of individual specimens is of limited use when dealing with cryptic species such as the An. gambiae complex. However, the study of the taxonomy is facilitated when the studies are made using populations and samples which have been defined by biochemical and cytological methods.

This thesis is dedicated to my mother.

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As this study was based on wild material it was not always possible for me to do the field collecting myself. Many people supplied me with live material and in particular I thank the following: Harold Cross, Neethling du Toit and Gideon van Eeden, National Institute for Tropical Diseases, Tzaneen; Keith Newberry and Dom Jan Jansen, National Institute for Tropical Diseases, Eshowe; Richard Hunt, S.A.I.M.R., for both wild material and the few colony specimens used.

I thank the following for access to museum specimens and co-operation during my visits to the various institutions: Dr. B.A. Harrison, Walter Reed Biosystematics Unit, National Museum (Natural History) Washington DC, U.S.A; Dr. M. Wood, Biosystematics Research Institute, Ottawa, Canada; Dr. P. Cranston, British Museum (Natural History), London; Drs J. Lane and G.B. White, London School of Hygiene and Tropical Medicine.

The Wits Computer Centre is thanked for help with the multivariate discriminant function analysis.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

There are two major reasons why the Anopheles gambiae group of mosquitoes were chosen for this study. Firstly, the group includes major vectors in Africa of human disease pathogens of the genera Wuchereria and Plasmodium. This means that a simple but accurate means of identification of the various species within the group is of prime epidemiological importance and has practical significance for the control and study of the diseases. Secondly, the controversy surrounding the group, from the time it was first postulated that "gambiae" might be more than one species, needs to be put in perspective in the light of contemporary knowledge. Both these areas have already been dealt with to some extent, either in isolated studies of narrow fields of interest or in reviews of the literature. Probably the most significant piece of work done on the gambiae complex is that by Paterson (1968) in an unpublished thesis. This will be dealt with in some detail later.

Since the last review (White, 1974) many changes have occurred. As theoretical concepts have altered and many new techniques have been introduced, this requires that the group once again be subjected to critical consideration. Certain faux pas that workers have made in their studies of member species need to be analysed so that we may benefit from their experience.

The main body of this thesis has as its aim a detailed and comprehensive examination of the gross morphology of the gambiae complex. New techniques and approaches are used in an attempt to find differences that are useful in the practical identification of mosquitoes in the field.

1.2 Species Complexes in the Genus Anopheles

In the early 1920's the first indications appeared pointing to the existence of species complexes. It was noticed that in some parts of Europe there was a curious absence of malaria where the common vector Anopheles maculipennis (Meigen) was abundant ("Anophelism without malaria"). It was eventually shown by improved taxonomic procedures that in fact maculipennis comprises at least six sibling species and that patterns on the egg chorion could be used to

separate them. Of these six species, two were discovered to be important vectors and a third was of minor importance. This explained the situation above of 'Anophelism without malaria". Bates (1949) reviewed all the information known at that time.

Essentially, the An. maculipennis complex was first resolved using behavioural and morphological characteristics. Later, the application of techniques such as chromosome cytology and electrophoresis, to test genetical concepts of species, revealed that species complexes are indeed rather common in anophelines. For example, in the Oriental region Anopheles maculatus consists of three species (Green et al., 1985a), culicifacies three species (Green & Miles 1980, Subbarao et al. 1983) and balabacensis three species (Baimai & Harrison, 1980). In Australia, Anopheles farauti is known to be a complex (Bryan 1970, Mahon et al. 1981) as is annulipes (Green 1972a). In the Afrotropical region the taxon Anopheles marshallii comprises four species (Lambert 1979, 1981), pharoensis two species (Miles et al. 1983), coustani two species (Coetzee 1982, 1983,, ziemanni two species (Coetzee 1982, 1984) and gambiae six species, to name just a few. Of the above, the gambiae complex is probably the most significant because it was the first to be resolved by applying a definite genetical concept of species using genetic approaches.

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It is also of interest because although a large amount of work has been done on the external morphology of the group, most of the species defy taxonomic separation, and genetical methods still have to be used to identify wild material. The members of this group are truly cryptic species.

1.3 Historical review of the Anopheles gambiae complex

It is now more than 80 years since Ross and co-workers (1900) discovered that the mosquitoes which today are known as members of the Anopheles gambiae Giles complex, were highly efficient vectors of human plasmodia and filarial parasites. The amount of literature published on the systematics of the group since then is immense and only a brief, though critical, resume is given below.

Although Anopheles gambiae was first described by Giles in 1902, it was not until much later that the species corresponding to this description became known by this name. Up until 1924 when Christophers revived the name of gambiae, these mosquitoes were generally known under the name of Anopheles costalis Loew (1866). Dönitz (1902) rejected the name costalis on the grounds that the common species known as "costalis" did not correspond with the description

given by Loew. Theobald (1903) defended the name of costalis because "The species has been so long known as costalis by all the important medical men in Africa that endless confusion would ensue [should the name be changed]". Such was Theobald's authority that the name gambiae did not finally replace costalis until publication of Edwards' monograph in 1932. Today it is known that Dönitz was correct and Anopheles costalis Loew is probably attributable to a member of the series Paramyzomyia (Mattingly, 1977).

For the next thirty years taxonomic publications on "gambiae" were rather limited and dealt mainly with variation noted in adults (eg. Evans 1938, De Burca & Yusaf 1942, De Meillon 1947, Holstein 1949, Hanney 1958). However, numerous papers were being published on the differences noticed in the biology of the species. It was noted that the larval habitats varied from open, sunlit, freshwater pools (eg. De Meillon 1937, 1941, Evans 1938, Haddow et al. 1947) to underground cement-lined water tanks (De Meillon, 1938), shaded pools (Causey et al., 1943), marshes (Vincke & Parent, 1944), flooded, well vegetated islands (Parent & Demoulin, 1945) and pools with high salinity (eg. Evans 1931, Ribbands 1944, Muspratt in De Meillon 1947, Muirhead- Thomson 1951).

Similarly, the adult biology also proved to be very

variable. Although "gambiae" in many areas was largely endophilic and anthropophilic (Gordon et al. 1932, Barber et al. 1932, Symes 1932, Gibbins 1933, De Meillon 1941), as more data were collected it became evident that the extent of zoophily was often surprisingly high. The following records of percentage positive for human blood in house collections give some idea of the range: Ethiopia 57% (Corradetti, 1938); Kenya 71-78% (Symes 1932, Kauntze & Symes 1933) and 62-80% (Smith in Wilson, 1960); Pare area of Tanzania 41-86% (Smith in Wilson, 1960); Zimbabwe 37-70% (Bruce-Chwatt & Gockel, 1960); Burkina Faso 61-99% (Hamon et al. 1959); north Cameroun 83% (Cavalie & Mouchet, 1961). Collections from outside or from animal shelters usually showed a much lower proportion of human bloodmeals. In the Pare area of Tanzania, Smith (1958) concluded that over half the "gambiae" were feeding on cattle.

With the advent of residual insecticide spraying around 1947, a large number of studies were concerned with the resting behaviour of the species. Studies in East Africa showed that relatively few females left untreated houses after feeding (Muirhead-Thomson 1951, Gillies 1954, Smith in Wilson 1960). However, in parts of West Africa 4-98% were found to leave on the night of feeding (Gelfand 1955, Mouchet & Gariou 1957). Moderate numbers of "gambiae" could be collected

resting outside in Mali (Sautet & Marneffe 1943, Holstein 1952), Burkina Faso (Haron et al. 1959), northern Nigeria (Service, 1963), northern Cameroun (Cavalie & Mouchet, 1961), Kenya (Symes 1941, Smith & Draper 1959), Tanzania (Draper & Smith, 1957), Zimbabwe (Leeson 1931,) and Transvaal (De Meillon, 1934). Gillies (1956) in Tanzania caught more than 3000 females within ten days, resting in an artificial outdoor shelter.

Mastbaum (1954, 1957) was probably the first to speculate on whether residual insecticide spraying of houses caused "gambiae" to change its behaviour from endophilic and anthropophilic to exophilic and zoophilic, vector to non-vector.

The major breakthrough came in 1962 when Paterson, Davidson and Kuhlow individually published evidence showing that "gambiae" was a complex of species or forms, although an indication had already been provided by Muirhead-Thomson (1945, 1951). In all three cases, the evidence presented was the results of cross-mating experiments. Paterson (1962) and Kuhlow (1962) showed that the East African saltwater-breeding form was a distinct species. Davidson & Jackson (1962) showed that the freshwater- breeding "gambiae" consisted of two "mating types" forms A and B, but it was only later that these "forms" were accepted by

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Davidson as species (Paterson, 1964). Davidson (1962) completed all possible crosses between A, B and the two saltwater-breeding forms found in East and West Africa and showed them to be incompatible, i.e., the hybrid males were sterile.

It is interesting to note here that as far back as 1944/45 two authors had already shown that melas, the West African saltwater-breeder, was a separate species from freshwater gambiae (Ribbands 1944, Muirhead-Thomson 1945, 1947). Muirhead-Thomson (1947) actually cross-mated melas and gambiae and when he saw that the hybrid males were sterile, he rightly concluded that they were separate species. These pioneering works were either ignored or ridiculed with spurious arguments and "facts" (Bruce-Chwatt, 1950).

Paterson et al. (1963) reported the existence in southern Africa of a third freshwater member of the complex, form C. Later, Paterson (1964) showed that the three freshwater members co-exist sympatrically at Chirundu, Zambia, without hybridizing, thus contradicting earlier statements by Hamon (1963) and later Coz & Hamon (1964). He checked the sex ratio of the adults obtained from the egg batches from wild females, examined the male progeny for fertility and the larval polytene chromosomes for asynapsis. No abnormalities were noted in a sample of families from

174 wild inseminated females from this area of sympatry. He concluded that the three forms were mating positively assortatively and were, therefore, separate species.

The significance of this conclusion in the fight against malaria has been well argued by Paterson (1963a, b). Paterson's thinking and his arguments in favour of a species complex are summed up in his unpublished doctoral thesis (1968). This thesis gives a good critical review of the work published up to 1966 and a valuable insight into the concept of species complexes.

Identifying the species. The discovery that gambiae is a complex helped to explain the pronounced ecological and behavioural diversity of these mosquitoes, where populations seemed to vary their breeding places, resting sites and host preferences to suit the immediate circumstances. For example, there was the hypothesis that residual insecticide spraying of houses exercised a powerful selection pressure on indoor resting "gambiae". This supposedly caused them to change their behaviour and rest outdoors (Muirhead-Thomson, 1951). These speculations were proved incorrect when Paterson et al. (1963) discovered the outdoor resting, zoophilic population to be a different species (form C). Species C also happens

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be a non-vector of human malarial and filarial parasites (see White, 1974).

So obviously, the identification of the different species became extremely important. As laboratory cross-mating techniques are laborious and time-consuming, more convenient methods were sought.

The West African saltwater-breeding form had long been known as "variation melas" (Theobald 1903, Evans 1938, De Meillon 1947) and was described as a melanic "form" of gambiae. The tolerance of the larvae to high salinity distinguished melas from the freshwater-breeding species. Likewise, the East African saltwater-breeding species could be separated from the others by salinity tests (Muirhead-Thomson, 1951). The name merus Dönitz (1902) was first proposed by Paterson (1963, unpublished WHO/MAL document no. 421) and formally introduced for this member of the complex by Coluzzi (1964).

Coluzzi's (1964) comprehensive morphological study of the four members of the gambiae complex, A, B, melas and merus, revealed some characters for separating the saltwater-breeders from the freshwater-breeders. This had already been done to some extent by Ribbands (1944), Muirhead-Thomson (1951) and Paterson (1963 unpublished WHO/MAL document no. 421). Coluzzi (1964)

failed to find reliable characters for separating A and B, which are sympatric over a large area of their distribution.

Subsequently, many workers have attempted to find morphological differences between the three freshwater species (Ismail & Hammoud 1968, Zahar et al. 1970, Clarke 1971, White & Muniss 1972, Reid 1973, 1975a, b) and failed. Ramsdale & Leport (1967), Green (1971), Bryan (1980) and Bushrod (1981) tested existing structural characters for separating the members of the group and found that they were not always reliable.

Cytogenetic studies of the giant polytene chromosomes found in the salivary glands of the fourth stage larvae and the nurse cells of the adult female ovaries, showed that the banding sequences differed between the five species (Coluzzi & Sabatini 1967, 1968, 1969). Green (1970, 1972b) and Service (1970) showed the practical value of using these chromosomal differences for routine identification of wild material. Davidson & White (1972) and Hunt (1972) confirmed the presence of a new sixth species of the gambiae complex from Uganda. The presence of this species had been suspected from the work of Haddow et al. (1947). The crossing and chromosome characteristics were described by Hunt (1972) and

Davidson & Hunt (1973). This is probably the most accurate and common method used these days for identifying the members of the complex.

More recently, electrophoretic enzyme studies have shown that the differences in the banding patterns are diagnostic for separating the species (Mahon et al. 1976, Miles 1978, 1979). However, this technique requires elaborate laboratory equipment and advanced technical knowledge. Interpretation of electrophoretic results requires considerable expertise which limits its application in practical malaria work.

The naming of the species. Paterson (1968) discussed the formal naming of the members of the gambiae complex. White (1975) proposed the same names as those suggested by Paterson but used rather different arguments to justify his proposal. Discussions about naming the species ceased after Mattingly (1977) published an article assigning the names suggested by Paterson nine years previously.

The East African saltwater-breeder was assigned the name merus Dönitz as mentioned above, originally suggested by Paterson (1963 unpublished WHO/MAL document no. 421) and later supported by Coluzzi (1964). Kuhlow (1962) described his saltwater-breeder as a new species Anopheles tangensis and this was sunk

into synonymy as merus had priority (Paterson 1963, loc. cit.).

The name melas Theobald was retained for the West African saltwater-breeder even though the holotype could not definitely be identified according to the parameters set down by Coluzzi (1964).

Species A was assigned the name gambiae Giles as the type specimen appears to be a freshwater-breeding member of the complex (Mattingly 1977) and no specimens of species B were found at the type locality by Gillies (in Mattingly, 1977) although this is not crucial evidence. Species C and D are also ruled out on present day distributional evidence. Anopheles gracilis Dönitz (1902) was sunk into synonymy because it is thought that that description was published after gambiae Giles (1902).

Species B is now known as arabiensis Patton (1905) due to the fact that it is the only species of the complex found in the Aden hinterland which is the type locality of arabiensis.

Species C has been assigned the name of quadriannulatus Theobald (1911). The type specimen was collected at Onderstepoort on the Transvaal highveld, South Africa, and as species C has been collected at high altitudes (see White, 1974) it is possible that

it is this species (Mattingly, 1977). However, more convincing evidence can be found in the 1939 Annual Report of the South African Institute for Medical Research, where Dr. B. de Meillon found "gambiae" on the Witwatersrand, Transvaal highveld, resting inside dairy stables and feeding on cattle.

Species D, known only from Bwamba, Uganda, and a mineral-water breeder, is considered to be a new species and has been named Anopheles bwambae (White, 1985).

Throughout the rest of this thesis "gambiae" will refer to An. gambiae sensu stricto, species A of the complex, unless otherwise stated.

CHAPTER TWO

SPECIES CONCEPTS

The Anopheles gambiae complex is a good example of how biological problems have been solved by applying a genetical species concept. Genetical concepts envisage "species" as real biological entities and not as artificial units of classification (as are the genera and higher categories). Because the gambiae complex has been resolved in genetical terms, for clarity it is appropriate that "species concepts" be considered in more detail. Enough has been written on species concepts to warrant a thesis, or even a monograph, on its own. In this chapter,I shall only go into enough detail to indicate the reasons for my preference for one concept above the others.

2.i The Taxonomic Concept

It is not possible to discuss species diversity in nature without the aid of classification and nomenclature. Aristotle can be considered the father of biological classification. He suggested the idea of "higher" and "lower" forms of life according to their

degree of perfection, which others translated into evolutionary terms after 1859 (see Mayr, 1969). Linnaeus is popularly considered the originator of taxonomy as we know it today. He introduced the binominal method of nomenclature and applied the Aristotelian system of logic to classification. This system was based on the morphological differences observed and the idea that species are divine creations. In a way, this can be called a species concept, however, it is an artificial taxonomic concept based purely on limited human observations of data perceivable by human senses and is not considered here.

Today we are aware of the conceptual distinctness of taxonomic species and genetical species, and it is becoming more generally accepted that there exist at least two kinds of genetical species. This is an important logical advance in Population Biology since this awareness is a first step towards avoiding the subtle nonsense generated by unwitting conflation of species concepts. There is a definite relationship between taxonomic species and genetical species which can be clarified; however, first it is necessary to consider the rival concepts of species in genetical terms.

Most biologists are in agreement that species are real

biological entities and not artificial categories recognised by man. However, there are two schools of thought on the genetical nature of species and how they arise. These are: 1) the Isolation Concept and 2) the Recognition Concept.

2.2 The Isolation Concept

Mayr (1969) defined species as being "groups of interbreeding natural populations that are reproductively isolated from other such groups". This definition of Mayr's is one of a long series of definitions by several biologists saying essentially the same thing, i.e., that "species" are defined in terms of their reproductive isolation from other species. That is, it is a relational concept.

Dobzhansky (1937) believed it was through the action of "isolating mechanisms" that the species gene pool was delimited. In so doing, he initiated a line of thought which was to influence the majority of biologists concerned with species concepts and modes of speciation. This theory proposes that two distinct populations are reproductively separated by ad hoc characteristics called "isolating mechanisms".

Isolating mechanisms fall into two distinct categories

(Mayr 1963, p 92):

1) Premating isolating mechanisms

Seasonal and habitat isolation (potential mates do not meet).

Ethological isolation (potential mates meet but do not mate).

Mechanical isolation (copulation is attempted but no transfer of sperm takes place).

2) Postmating isolating mechanisms

Gamete mortality (sperm transfer takes place but the egg is not fertilized).

Zygote mortality (egg is fertilized but zygote dies).

Hybrid inviability (zygote produces an F1 hybrid of reduced viability).

Hybrid sterility (F1 hybrid zygote is fully viable but partially or completely sterile, or produces deficient F2 hybrids).

The use of the above criteria tends to direct thinking towards what happens when two species meet. Little emphasis is placed on the important question of how males and females of the same species behave when in contact with each other. It also implies that different species actually repel one another (Hammond, 1982). The term "species integrity" is often used

despite its "group selection" connotations. Dobzhansky (1976) viewed species as "not accidents but adaptive devices through which the living world had deployed itself to master a progressively greater range of environments and ways of living". This is startlingly similar to the theories of todays Creationist scientists (see Hitching, 1982), and is, to say the least, teleological.

Central to the isolation theory is the idea that natural selection plays a part in evolving isolating mechanisms. The process is supposed to follow the following course: two populations separate, diverge genetically to some extent and then come together again. Mating still occurs between the two populations but to some extent the hybrids are disadvantaged (not viable, sterile, ill-adapted, etc.). Natural selection then favours individuals that mate only with their own group and thus reinforces their incipient isolating mechanisms (Ayala et al. 1974). (However, assuming that the two populations have diverged in allopatry to such an extent that their subsequent overlap produces disadvantaged hybrids suggests that natural selection could not have produced reproductive isolating mechanisms.) Central to the theory of speciation by reinforcement is the idea that isolating mechanisms arise under selection in sympatry with a closely related species, and that in allopatry these

characters somehow diffuse through the non-sympatric part of the population despite their disadvantages. Mayr (1963) states "Where no other closely related species occur, all courtship signals can 'afford' to be general, nonspecific and variable. Where other related species coexist, however, nonspecificity of signals may lead to wasteful courtship and delays, even where no hetero-specific hybridization occurs. Under these circumstances there will be a selective premium on precision and distinctiveness of signals." The first sentence implies that the role of reproductive behaviour in leading to fertilization does not matter. This clearly reveals that Mayr regarded isolating mechanisms as true adaptations as defined by Williams (1966).

2.3 The Recognition Concept

In 1978 a publication appeared which seriously questioned the theory of isolating mechanisms and reinforcement (Paterson, 1978). Using the evidence that other authors used to support the isolation theory, Paterson demonstrated the flaws inherent in their experiments and arguments. For example, laboratory experiments claiming to show empirical support for reinforcement had actually been designed in such a way as to eliminate the outcome to be expected under population genetic theory of negative

heterosis. When two populations of a species with different recessive genetical markers were placed in the same cage and allowed to interbreed, instead of allowing the experiment to proceed to its natural conclusion, the numbers of the two marked groups were artificially kept at equality after each generation (eg. Crossley, 1974). Thus was reinforcement reinforced! Paterson (1978) pointed out that such interference is unacceptable and does not support the reinforcement theory. This criticism has subsequently been empirically supported by Harper & Lambert (1983).

Having for these and many other reasons rejected the "Isolation Concept" of species, Paterson provided a satisfactory alternative. Paterson's (1985) definition of a species is "...that most inclusive population of individual, biparental organisms which share a common fertilization system." In motile organisms, individuals of a population in their preferred or normal habitat share a common specific-mate recognition system (SMRS) which is a necessary preliminary to fertilization. The SMRS comprises a co-adapted signal-response reaction chain whose function it is to ensure fertilization under the usual conditions of the species preferred habitat. The SMRS may take the form of visual, auditory, chemical, tactile or any other signals and responses (or combination of these signals) exchanged between

potential mates or their cells. The SMRS is best developed in motile organisms, and plays a much less important role in sessile animals and plants (Paterson, 1985). For example, in orchids the SMRS is restricted to the interaction between the pollen and stigma. The rest of the fertilization system is what determines the limits of the gene pool, i.e., the signalling between plant and pollinator.

Using Paterson's Recognition Concept, no mechanisms are necessary to protect species "integrity". The concept involves a line of thought directed entirely at a single population (species), and explains how the individual within that population behaves and what are the limiting factors for gene flow. It is a non-relational concept, in contrast to the Isolation Concept.

In considering how new species may arise, Paterson (1985) states "...speciation is an incidental effect resulting from the adaptation of the characters of the fertilization system, among others, to a new habitat, or way-of-life." While members of a species remain in their normal habitat the characters of the fertilization system are maintained under stabilizing selection. When a small group of conspecific individuals become isolated from the main population, less well adapted characters to the new habitat,

including fertilization characters, will become subject to directional selection. The new fertilization system would set the limits for gene recombination. Should the fertilization system then differ sufficiently from the parent population, speciation can be said to have occurred. Although this model of speciation is very similar to Mayr's (1963) view of geographic speciation it is not identical. Paterson (1985) states "Because Mayr conceives species in terms of reproductive isolation, he is obliged to invoke the pleiotropic modification of 'isolating mechanisms' in allopatry to account for geographic speciation." This is not a problem using the Recognition Concept of species as speciation results from the adaptation of fertilization characters to the conditions in the new habitat.

2.4 Discussion

It may seem, at first glance, that these two concepts are mutally correlated and to split them is splitting hairs. This is not so, although Hammond (1982) sees no difficulty in fusing them when he states "Attractants then may play an important role in providing greater opportunities for homogametic matings but, to the extent that they are specific, may also be involved in the reproductive separation of populations. Generally speaking specific attractant signals may be regarded

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as contributing to reproductive barriers which are of a mate 'avoidance' type." What Hammond seems to fail to realise is that if we are to understand how evolution occurs, we must be sure to distinguish "adaptations" from incidental "effects" as Williams (1966) so carefully explained. The point is that there is no evidence that any of the "isolating Mechanisms" are ad hoc characters (i.e., adaptations s.str.) as Mayr claims. They "isolate" purely incidentally. There is no evidence that they were selected to fulfil the role of preserving the integrity of the species. It is the confusion of "adaptations" with "effects" that Hammond seems intent on doing.

Paterson (1985) gives very good reasons why the two concepts of Recognition and Isolation are mutually exclusive. He argues that the isolation concept is not compatible with the allopatric mode of speciation. How, he asks, are isolating mechanisms, as ad hoc characters, presumed to arise in a situation of total allopatry, eg. on islands? Mayr (1963), an ardent proponent of speciation in allopatry, does not adequately answer this question. In his discussion, Paterson concludes that "...all phenomena covered by the category 'postmating isolating mechanisms' (Mayr 1963) are incidental to delineating species, since they have nothing to do with bringing about fertilization". Many more arguments are presented in

favour of the recognition concept and against the isolation concept.

In considering Darwin's ideas on the origin of species Paterson (1985) states "...Darwin's view of speciation was detailed enough for us to see that he accepted species arise as incidental consequences of adaptation". He goes on to quote Kuhn (1970)

> "For many men the abolition of that teleological kind of evolution was the most significant and least palatable of Darwin's suggestions. The Origins of Species recognised no goal set either by God or Nature."

and then says "Thus, in sharp contrast to the Isolation Concept, the Recognition Concept is in complete accord with the revolutionary view of Darwin. Moreover, the Recognition Concept emphasizes the incidental nature of speciation and expresses it in genetical terms, besides providing a genetical concept of species."

In this thesis the genetical concept followed is the Recognition Concept as a basis for delimiting the field for gene recombination in the populations under study.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

A large section of this chapter would normally have been included only as an appendix. However, there are certain important modifications to techniques which form the basis of this project. I have, therefore, placed all the relevant information on collections and laboratory methods in one chapter.

3.2 Field collections

Female mosquitoes, identified morphologically as belonging to the *Anopheles gambiae* complex (Gillies & De Meillon, 1968) were collected by various means from numerous localities. A summary of this data is presented in Table 1. Collecting sites are mapped in Figure 1.

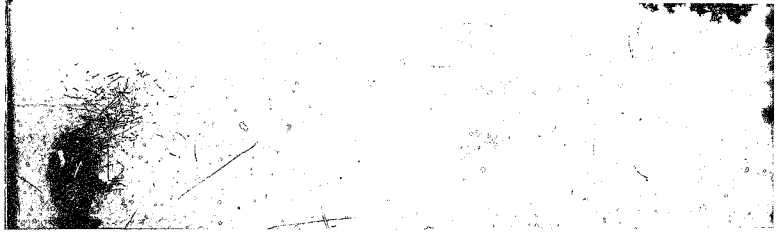


TABLE 1. Details of collections of members of the Anopheles gambiae group of species.

Species	Locality	Map Reference	Collection Methods	No. wild Adults	No. wild Larvae	Total No. Adults
gambiae	Mahongo, Namibia	18°05'S, 21°45'E	Resting indoors	7		48
	Yaka Yaka, Congo	4°23'S, 15°09'E	" "	44		135
	Grand Comoros	11°40'S, 43°16'E	Larval collection		115	60
				51	115	243
arabiensis	Pafindaba, Zululand	27°05'S, 32°33'E	Man-baited net	17		29
			Cattle kraal	7		7
			Biting man outdoors	5		6
	Tetepan, Zululand	27°02'S, 32°15'E	Knockdowns	2		2
	Komatipoort, Transvaal	25°26'S, 31°56'E	Biting man outdoors	2		8
			Pit collection	2		9
	Jaffray, Transvaal	23°50'S, 30°20'E	Biting man outdoors	1		3
	Nsoze, Swaziland	26°40'S, 31°36'E	" " "	3		12
	Big Bend, Swaziland	26°49'S, 31°56'E	" " "	1		1
	Mahongo, Namibia	18°05'S, 21°45'E	Resting indoors	3		9
	Kanyemba, Zimbabwe	15°40'S, 30°20'E	Biting man outdoors	30		30
				74		118
merus	Kosi Bay, Zululand	26°55'S, 32°55'E	Cattle kraal	1		10
	Opansi, Zululand	27°34'S, 32°18'E	" "	39		105
			Biting man outdoors	3		13
	Makanis Drift, Zululand	27°02'S, 32°19'E	Pit collection	2		20
			Cattle kraal	9		9
	Shemula, Zululand	27°05'S, 32°17'E	Pit collection	2		20
			Cattle kraal	2		20
	Pafindaba, Zululand	27°05'S, 32°33'E	Man-baited net	1		1
	Tugela River Mouth, Zululand	29°20'S, 31°20'E	" " "	1		5
	Mhandini, Zululand	27°50'S, 32°10'E	Pit collection	14		18
	Soutini, Transvaal	23°26'S, 30°56'E	Biting man outdoors	3		22
			Larval collection		3	3
	Riland, Transvaal	23°35'S, 30°31'E	" "		4	4
				77	7	250
quadriannulatus	Constantia, Transvaal	23°35'S, 30°35'E	Pit collection	4		37
	Roosmond, Transvaal	23°20'S, 30°10'E	Cattle kraal	1		10
	Komatipoort, Transvaal	25°25'S, 31°56'E	Man-baited net	5		29
			Pit collection	8		60
	Duiwerl, Transvaal	23°48'S, 30°10'E	Larval collection		2	2
	Soutini, Transvaal	23°26'S, 30°56'E	"		6	6
	Nzeki, Transvaal	23°10'S, 30°35'E	"		9	9
	Shemula, Zululand	27°05'S, 32°17'E	Pit collection	2		20
	Opansi, Zululand	27°34'S, 32°18'E	Cattle kraal	4		11
			Pit collection	1		1
	Makanis Drift, Zululand	27°02'S, 32°19'E	" "	3		26
			Cattle kraal	5		5
			Larval collection		12	12
	Umfolozi River, Zululand	28°20'S, 32°20'E	" "		19	19
	Pafindaba, Zululand	27°05'S, 32°33'E	Cattle kraal	2		2
	Kanyemba, Zimbabwe	15°40'S, 30°20'E	Biting man outdoors	1		1
				36	48	260

FIGURE 1.

A map of Africa, south of the Equator, showing grid localities of collection sites. Some collection sites occupy the same grid square.

Live wild females were all subjected to the following laboratory procedures. Blood fed and gravid females were individually isolated in small tubes containing damp filter paper to induce egg-laying. Unfed females were offered a blood meal before being isolated. Females which laid eggs and survived were re-fed for chromosomal identification (see 3.4). Those which laid eggs and died were stored in liquid nitrogen for electrophoretic identification (see 3.5). In certain cases, some of the F-1 progeny were used for both chromosomal and electrophoretic identifications. Some wild females were identified without obtaining egg batches.

3.3 Morphological techniques

Each egg batch obtained in the laboratory was treated as follows. The egg batches were placed in distilled water in individual plastic bowls. The emerging larvae were fed on a mixture of powdered dog biscuits and brewers yeast until they reached late fourth stage development. At this point the larvae were individually isolated in small tubes. At pupation and emergence of the adult, the discarded larval and pupal pelts were collected and stored in 80% alcohol. The immature pelts were later mounted in phenol/alcohol/ Canada balsam (Wirth & Marston, 1968). The adults were

glued onto card triangles held on insect pins. This method of rearing mosquitoes ensured that immature pelts and adults were correlated for every single individual used in this study.

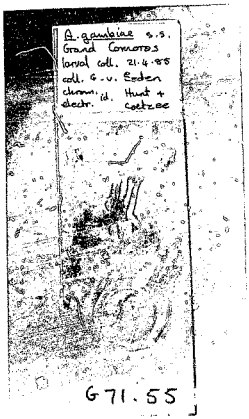
On a few occasions wild larvae were obtained from the field and these were treated in the same way as the F-1 larvae were. However, once the adults emerged they were not killed and pinned out. Instead, an attempt was made to have each individual identified either chromosomally or electrophoretically.

Adults used only for electrophoresis were kept alive for 24 hours and then anaesthetized with ether. The wings, legs and palps were carefully removed from the body and dry mounted on a microscope slide (Fig. 2). The body was then stored in liquid nitrogen for later electrophoretic examination.

Adults obtained from the larvae collected on the island of Grand Comoros were subjected to a more complicated routine as a correlated chromosomal and electrophoretic identification was required. Obtaining half gravid ovaries from virgin females is extremely difficult. Each female resulting from the larvae was isolated with ten Anopheles gambiae colony males for four to five days and fed on sugar-water during this time. The females were then starved overnight and

FIGURE 2.

Photograph of a slide with wings, palps and legs dry-mounted, the coverslip being held by mountant at the corners only. Cleared antennae have been mounted in Faure's gum chloral and the coverslip ringed with Entellan. The slide is suitably labelled for a museum collection.


A. gambiae s.s.
Grand Comoros
larval coll. 21.4.88
coll. & v. Sedon
chrom. id. Hunt +
electr. Coetzee
G71.55

offered a blood meal the next morning. Where ovarian development took place, the females were dissected, their wings, legs and palps were dry mounted, and the ovaries preserved in Carnoy's fluid for chromosomal studies. The remains of the bodies were stored in liquid nitrogen for electrophoresis.

The males emerging from the Grand Comoros larvae were kept alive on sugar-water for at least five days and were then used in attempts at artificial mating (Baker et al. 1962). Prior to mating, the wings, legs and palps were removed from the males and dry mounted. After mating attempts, the bodies were stored in liquid nitrogen for electrophoresis. One successful mating between a Comoros male and gambiae colony female was achieved and the offspring were identified using both chromosomal and electrophoretic techniques, and the internal male genitalia were examined for sterility.

Adults were examined under a stereo microscope at 50X magnification. Certain spots on the wings, legs and palps were measured with a micrometer eyepiece. Female antennae were cleared in 10% potassium hydroxide and mounted in Faure's gum chloral (Gatenby & Beams, 1950) for examination of the coeloconic sensilla. Specimens used for scanning electron microscopy were acetone-dried (Truman, 1968) and mounted directly on stubs

with double-sided sellotape and sputter-coated once with gold to a thickness of 20nms.

Setal counts of the larvae and pupae followed the system of Belkin (1962) using a phase-contrast microscope at 400X magnification.

Hatched eggs were preserved in alcohol, then air-dried and mounted for scanning electron microscopy.

3.4 Chromosomal identification

Half gravid ovaries (Christopher's 1911, stage III) were dissected from wild or F-1 progeny females. The terminal segments of the abdomen were grasped with fine forceps and the ovaries were pulled out of the body while gently squeezing the mosquito between thumb and forefinger. They were immediately placed in Carnoy's fixative (3 parts ethanol, 1 part glacial acetic acid) and left for at least 48 hours (Hunt & Coetzee, 1986a).

Chromosome preparations were made using the techniques of Hunt (1973) and Green & Hunt (1980). Ovaries were removed from Carnoy's and placed in a drop of 50% propionic acid on a microscope slide. After clearing (±30 sec.) the ovaries were broken up with dissecting needles and a drop of lacto-acetic-orcein added. The

ovarioles were stained for ±7 minutes. A drop of 50% propionic acid was added and a coverslip dropped on top. The coverslip was tapped to break the nuclei and release the chromosomes. The slide was then blotted with filter paper, taking care not to move the coverslip.

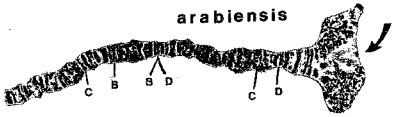
Identification of the species was obtained using simplified chromosomal maps (Figs. 3, 4). Photographs of the chromosomes, both as a record of identification and to produce the maps, were taken on a Vickers phase-contrast microscope (X1000) using Kodak Technical Pan black and white film (ASA 50). The negatives were developed in Kodak H110 for 8 minutes at 70°C. Prints were developed in Kodak D163 for 2 minutes.

3.5 Electrophoresis

Initial electrophoretic identifications were carried out using the techniques outlined by Mahon et al. (1976) and Miles (1978). This involved the use of starch as a gel matrix and the slicing of the starch into 3 horizontal layers so that the enzyme systems superoxide dismutase (SOD), octanol dehydrogenase (ODH), glutamic-oxaloacetic transaminase (GOT) and an-specific esterase (EST) could be stained for.

FIGURE 3.

Chromosomal map of the X chromosomes showing breakpoints of inversions used to identify *Anopheles arabiensis*, *quadriannulatus*, *gambiae* and *merus*, with *quadriannulatus* as the standard arrangement. The arrows indicate the centromere end of the chromosomes.


arabiensis
C
B
S
D
C
D


quadriannulatus

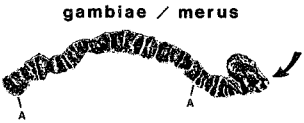
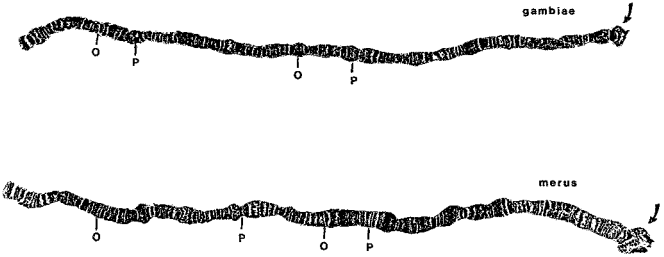

gambiae / merus
A
A

FIGURE 4.

Chromosomal map of arm 2 showing inversions o and p which are used to separate Anopheles gambiae from Anopheles merus. Arrows indicate the centromeres.


gambiae
O
P
O
P
merus
O
P
O
P

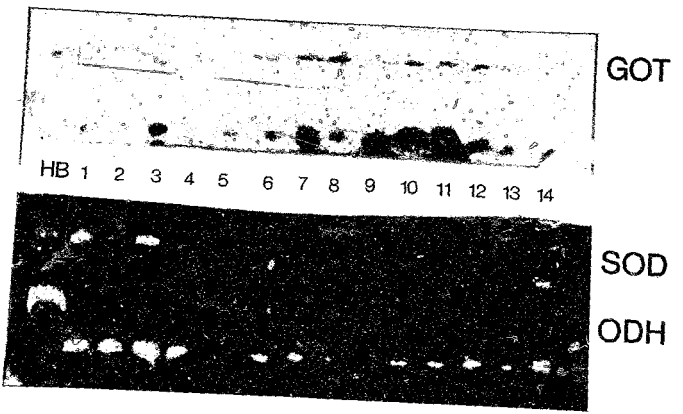
Later modifications to the technique using stacked polyacrylamide gels (Hunt, 1984) resulted in better resolution of the electromorph bands (Fig. 5). Variation within gambiae was observed at the GOT locus in the Grand Comoros sample (Hunt & Coetzee, 1986b), and the SOD locus in the Yaka Yaka sample (Hunt & Coetzee, in prep.). However, this did not affect the usefulness of the systems for the identification of certain species of the complex (Miles, 1979).

The SOD/ODH staining method used for starch gels (Mahon et al., 1976) was not effective when using acrylamide gels and had to be modified. Gels were placed in a staining dish containing a solution of 50ml 0.05M Tris/HCl buffer pH 8.5, 25mg nicotinamide adenine dinucleotide, 50mg nitro-blue tetrazolium, 5mg phenazine methosulphate, 1ml ethanol and 0.2ml 2-octanol. The staining dish was then covered with clear plastic and floated on a 37°C waterbath for 2-3 hours in daylight or until both ODH and SOD bands appeared. The gel was then transferred to a staining dish containing only distilled water and left overnight before fixing in 7% acetic acid.

Discussion of the use of chromosomes and electrophoresis for the identification of the gambiae complex is in Appendix IV.

FIGURE 5.

Polyacrylamide gels showing the electromorph banding patterns used to identify the four species Anopheles gambiae (2, 4-13) and arabiensis (1) (ODH), quadriannulatus (3) (GOT) and merus (14) (SOD). HB = human blood marker.


GOT
HB 1 2 3 4 5 6 7 8 9 10 11 12 13 14
SOD
ODH

3.6 Discussion

Up until now it has not been demonstrated that it is possible to correlate chromosome and electrophoretic data with pinned museum specimens. That is, museum specimens have either originated from identified laboratory colony stocks or, rarely, from progeny of identified wild material. The methods described above allow an accurate assessment of the morphological variation within populations without subjecting the individuals to excessive laboratory pressures.

In the present study I decided not to use larval polytene chromosomes (found in the salivary glands, Coluzzi & Sabatini, 1967) as a means of identification because this technique does not allow the preservation of morphological specimens. As this study is concerned mainly with the identification of malaria vectors (as should all malaria control programmes) the destruction of the larvae would be counter-productive.

Electrophoretic enzyme variation displayed on polyacrylamide gel matrices indicates that more detailed studies are needed on this group of species. The variation seen at the fast (100) GOT locus in gambiae may indicate that this species is polymorphic for this enzyme system. However, it was not possible to establish with certainty whether some individuals

were heterozygous as the bands were too diffuse. Two individuals of gambiae from Brazzaville were heterzygous for the 105/100 loci. The 105 locus was previously considered to be species-specific for bwambae which is only found in Uganda (Miles, 1979).

The combined techniques for morphological specimens correlated with cytogenetic and electrophoretic identification methods have been published by Hunt & Coetzee (1986).

CHAPTER FOUR

RESULTS

4.1 *Adults*

Several morphological characters have been examined including those reported on by previous workers (Coluzzi 1964, Ismail & Hammoud 1968, Coetzee *et al.* 1982) and the results are given below.

The palp ratio (length of segments IV + V/III) was used by Coluzzi (1964) and Bryan (1980) in West Africa to differentiate merus and gambiae, and by Bushrod (1981) in East Africa to separate merus from gambiae/arabiensis. In the present study, 127 palpi were measured and the results are shown in Table 2. An. merus (0.83-0.94) has a ratio significantly higher than the other three species and 50% of the merus sample could be identified on this character alone. (See Table F, Appendix I)

Palps with three pale bands (Fig. 9) are most commonly seen in the freshwater members of the gambiae complex. The salt-water breeders have a higher proportion of

Table 2. Palpal ratios of four species of the gambiae complex.

Species	n	Range	Mean	S.D.
gambiae	24	0.674-0.859	0.76	0.05
arabiensis	30	0.699-0.882	0.79	0.04
quadriann.	52	0.711-0.867	0.79	0.04
merus	21	0.83-0.94	0.88	0.03

Degree of overlap.

	Range	%
gambiae/arabiensis	0.699-0.859	88.9
gambiae/quadriannulatus	0.711-0.859	90.8
gambiae/merus	0.83-0.859	11.1
arabiensis/quadriannulatus	0.711-0.882	97.6
arabiensis/merus	0.83-0.882	27.5
merus/quadriannulatus	0.83-0.867	17.8

4-banded palps (Davidson et al., 1967). The number of 4-banded palps were recorded with the following results: gambiae 0% (n=84); arabiensis 4.6% (n=86); quadriannulatus 26.9% (n=119); merus 75.2% (n=149).

Coeloconic sensilla on the antennae (Fig. 6) were counted on 197 specimens and the results are presented in Table 3. An. merus has significantly more sensilla

FIGURE 6

A scanning electron micrograph of antennal flagellum segment 3 showing the coeloconic sensilla (mag. X2200).

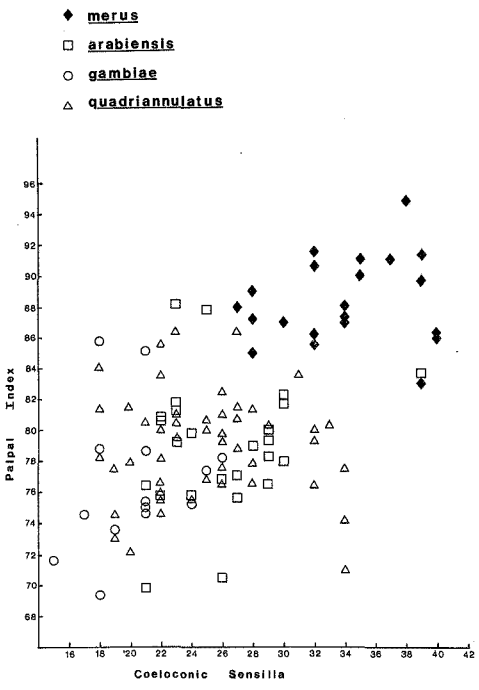


Table 3. Number of coeloconic sensilla on the antennae of four species of the gambiae complex.

Mean no./ flagellar segment	gambiae n=65	arabiensis n=41	quadri-annulatus n=47	merus n=44
1	2.3	2.6	2.5	4.3
2	3.6	4.2	3.6	5.2
3	4.1	4.4	4.2	6.0
4	3.4	4.1	3.7	5.7
5	2.9	3.7	3.8	5.2
6	2.3	3.2	2.8	4.1
7	1.9	2.1	2.4	3.1
8	0.5	0.9	0.6	0.4
9	0.3	0.6	0.5	0.4
10	0.02	0.2	0.02	0.07
11	0	0	0	0
12	0.03	0.02	0	0
13	0	0	0	0
1 + 2	5.9	6.8	6.1	9.5
Total	21.2	26.2	24.8	34.5
Range	15-30	21-39	18-34	24-41

FIGURE 7

Scatter diagram using the palpal index and the number of *coelaconic sensilla* showing the separation of Anopheles merus from the other three members of the gambiae complex.


merus
arabiensis
gambiae
quadriannulatus
Palpal Index
Coeloconic Sensilla
96
94
92
90
88
86
84
82
80
78
76
74
72
70
68
16
18
20
22
24
26
28
30
32
34
36
38
40
42

than the other three species with a mean total of 34.5 (see Table E, Appendix I). This agrees generally with the results of Ismail & Hammoud (1968) although their mean total values differ slightly from those given here.

Using Bushrod's (1981) combination of palp ratio and coeloconic sensilla number, a graph was plotted (Fig. 7) which shows *merus* as being quite distinct from the others.

During a scanning electron microscopic study of the antennae, it was noticed that *merus* had more spicules on the bases of flagella segments 3 and 4 (Fig. 8) than did the other species. As this character was extremely difficult to quantify, the following table is only an indication of the number of spicules present per species.

Table 4. Spicules on antennal segments.

Species	Flagellum segments				
	1	2	3	4	5
gambiae	+++	++	+	-	-
arabiensis	+++	++	+	-	-
quadriannulatus	+++	+	±	-	-
merus	+++	++	++	+	±

FIGURE 8

Basal antennal segments showing the minute spicules at the bases of the segments (mag. X850).



The size of wing spots (Fig. 9) and their presence or absence were recorded. Statistical analyses of these spots are given in Tables A and B of Appendix I. Although the mean size of several spots showed significant differences, these were not sufficiently large to be of use for easy identification purposes. The presence or absence of spots had no taxonomic significance.

Hind leg banding patterns (Coetzee et al., 1982) were first assessed subjectively, i.e., overlapping the segmental joints or not (Fig. 10), and subsequently subjected to quantitative analysis (Coetzee, 1986). Measurements were taken of the pale bands at the joints of hind tarsomeres 3/4 and 4/5 (n=806). Figure 11 shows the amount of overlap in the size of the bands on gambiae/arabiensis and merus/quadriannulatus. 95% of all specimens examined here could be grouped using this character. Statistics are given in Tables C and D of Appendix I.

Scanning electron microscopy (SEM) studies of the tarsal claws (Fig. 12) were conducted but no differences between the species could be detected.

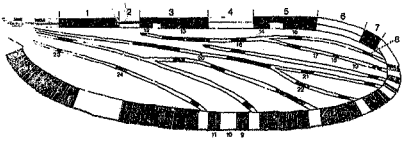
Similarly, both SEM and light microscopy studies of the male genitalia revealed no obvious differences between the species.

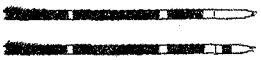
FIGURE 9

Line drawing showing wing spots and palpal bands which were measured or recorded for presence or absence.

FIGURE 10

Hind leg bandings used to discriminate *gambiae*/*arabiensis* from *merus*/*quadriannulatus*.







gambiae / arabiensis



merus / *quadriannulatus*

FIGURE 11

Distribution of the leg banding measurements of gambiae/arabiensis (dotted line) and merus/quadriannulatus (solid line).

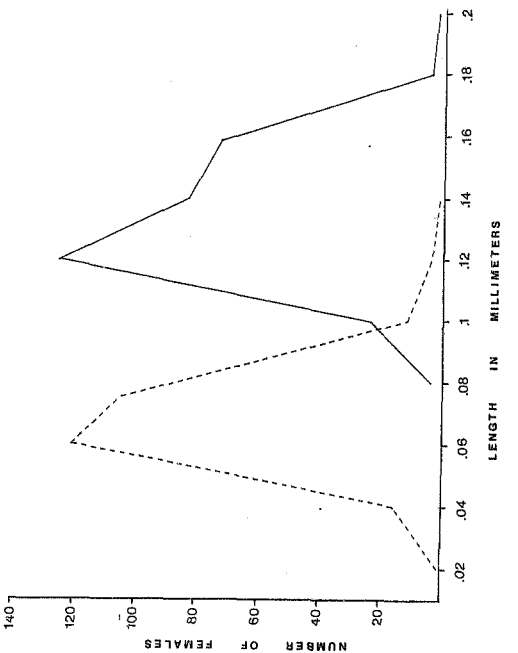


FIGURE 12

Scanning electron micrographs (mag. X1100) of the fore tarsal claws of *Anopheles gambiae*, a) male, b) female.



A



B

4.2 Pupae

Full setal counts (Belkin, 1962) were done on at least 10 individuals per species. This entailed recording the number of branches for 222 setae per pupa (Fig. 13). Setae which showed some differences were examined further and the number of branches recorded for the rest of the sample. Combinations of setal counts devised by Coluzzi (1964) and Reid (1975a, b) were also recorded. These results are given in Tables 5-7.

Table 5. Statistical analysis of the number of branches on 11 pupal setae.

Seta	Species	n	Range	Mean	S.D.
10-C	gambiae	91	1-4	2.30	0.72
	arabiensis	65	1-6	2.77	0.93
	quadriannulatus	60	1-5	2.42	0.79
	merus	59	2-5	2.86	0.73
4-I	gambiae	83	4-9	6.08	1.20
	arabiensis	55	4-9	6.04	1.04
	quadriannulatus	51	4-10	6.67	1.24
	merus	58	3-8	5.41	0.97

Table 5. cont.

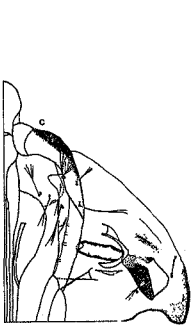
Seta	Species	n	Range	Mean	S.D.
5-I	gambiae	85	1-3	2.29	0.63
	arabiensis	58	1-4	2.69	0.57
	quadriannulatus	56	1-4	2.82	0.47
	merus	51	1-3	1.98	0.51
7-I	gambiae	79	1-9	4.37	1.65
	arabiensis	56	2-7	4.64	1.15
	quadriannulatus	53	2-7	5.09	1.38
	merus	55	3-11	5.96	1.75
4-II	gambiae	93	3-8	5.19	1.37
	arabiensis	61	3-8	5.38	1.13
	quadriannulatus	58	4-9	6.34	1.25
	merus	62	3-6	4.48	0.92
6-III	gambiae	87	1-5	2.29	0.90
	arabiensis	69	1-5	2.96	0.86
	quadriannulatus	50	1-5	2.78	0.68
	merus	52	1-6	2.42	1.11
6-IV	gambi ae	80	1-3	1.69	0.61
	arabiensis	67	1-2	1.54	0.50
	quadriannulatus	49	1-3	1.71	0.58
	merus	52	1-2	1.62	0.49

Table 5. cont.

Seta	Species	n	Range	Mean	S.D.
3-V	gambiae	93	1-3	1.12	0.36
	arabiensis	68	1-3	1.28	0.48
	quadriannulatus	59	1-2	1.02	0.13
	merus	60	1-2	1.15	0.36
6-V	gambiae	91	1-3	1.62	0.55
	arabiensis	69	1-3	1.58	0.55
	quadriannulatus	49	1-2	1.53	0.50
	merus	55	1-2	1.20	0.40
3-VI	gambiae	94	1-2	1.09	0.28
	arabiensis	72	1-2	1.11	0.32
	quadriannulatus	62	1-3	1.10	0.35
	merus	61	1-2	1.02	0.13
7-VII	gambiae	97	1-3	1.18	0.41
	arabiensis	69	1-2	1.28	0.45
	quadriannulatus	59	1-2	1.02	0.13
	merus	58	1-2	1.02	0.13

FIGURE 13

Line drawing of a pupa showing the setal numbering system of Belkin (1962). Dorsal setae are on the left and ventral setae on the right. (From Coetzee & Du Toit, 1979)


C

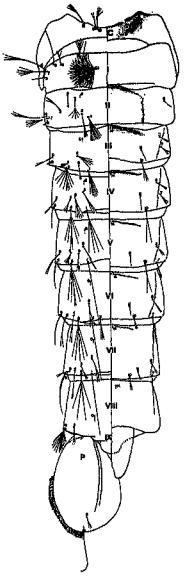

E
I
II
III
IV
V
VI
VII
VIII
P

Table 6. Statistical analysis of setae combinations with Coluzzi's (1964) values in parenthesis. Species names have been abbreviated.

Setae	Species	n	Range	Mean	S.D.
4+5,II	gamb.	50 (140)	12-22 (13-25)	16.80 (17.84)	2.89 (2.27)
	arab.	35 (100)	10-21 (11-24)	17.03 (16.22)	2.52 (2.58)
	quad.	31	15-25	19.13	2.68
	merus	31 (40)	10-19 (10-16)	14.42 (13.33)	2.13 (1.37)
1,III+IV	gamb.	50 (140)	9-21 (9-17)	14.60 (13.03)	2.19 (1.54)
	arab.	37 (100)	10-24 (12-24)	16.92 (16.32)	3.34 (2.49)
	quad.	31	10-21	15.39	2.56
	merus	31 (40)	10-19 (10-30)	13.94 (17.38)	2.50 (4.59)
2,I+II+ III	gamb.	49 (140)	24-40 (22-45)	32.02 (31.61)	4.13 (4.20)
	arab.	35 (100)	24-39 (24-39)	31.74 (30.51)	3.60 (2.95)
	quad.	31	23-40	29.74	4.43
	merus	31 (40)	21-36 (25-40)	29.58 (30.10)	3.30 (3.15)
2,IV+V+ VI+VII	gamb.	49 (140)	21-39 (21-36)	28.90 (26.04)	3.64 (2.31)
	arab.	36 (100)	21-37 (22-35)	28.94 (27.50)	4.00 (2.77)
	quad.	31	23-40	27.35	3.99
	merus	31 (40)	21-31 (19-26)	25.16 (22.95)	2.57 (1.74)

Table 6. cont.

Setae	Species	n.	range	mean	S.D.
4,II+2,V +10,VII	gamb.	48 (140)	18-33 (18-30)	23.94 (22.68)	3.26 (2.35)
	arab.	33 (100)	18-31 (18-32)	24.61 (23.23)	2.88 (2.74)
	quad.	31	20-34	26.16	3.30
	merus	31 (40)	17-26 (14-21)	20.55 (17.73)	2.26 (1.81)

Table 7. Statistical analysis of setae combinations proposed by Reid for East Africa (1975a) and West Africa (1975b).

Setae	Species	n.	Range	Mean	S.D.
4,II-2,VII	gamb.	49	-1 - +10	4.24	2.64
	E.Afr.	43	3 - 9	5.50	
	arab.	35	1 - 8	4.37	1.75
	E.Afr.	31	-2 - +4	1.10	
	quad.	31	3 - 12	6.65	2.06
	merus	31	-1 - +6	3.00	1.63
9,VII-4,II	gamb.	50	8 - 30	16.52	5.33
	E.Afr.	39	0 - 22	13.20	
	W.Afr.	35	12 - 32	20.80	
	arab.	32	6 - 25	17.78	4.46
	E.Afr.	28	17 - 28	21.00	
	W.Afr.	34	-1 - +16	8.70	
	quad.	31	2 - 23	13.48	5.67
	merus	31	6 - 28	18.61	6.22
3,III	gamb.	50	2 - 7	2.96	1.23
	E.Afr.	44	2 - 5	2.50	
	arab.	38	2 - 4	2.55	0.86
	E.Afr.	27	2 - 7	4.30	
	quad.	30	2 - 5	2.80	1.00
	merus	31	2 - 7	4.13	1.09

The Student's t-test for difference between means was used for all the setae in Table 5 and seta 3-III in Table 7. The results are given in Table 8, Appendix I.

Differences in setal branching between arabiensis in Namibia and arabiensis in the Transvaal and Natal were compared with gambiae from Namibia. The frequencies of the number of branches of certain setae are shown in Table 8.

Table 8. Frequency of number of branches on some setae of gambiae and arabiensis from Namibia (W) and arabiensis from the Transvaal and Natal (E).

Seta	Species	No. branches					
10-C		1	2	3	4	5	6
	gamb.	.20	.43	.30	.07		
	arab. W			.33	.45	.11	.11
	arab. E	.02	.54	.35	.09		
4-I		4	5	6	7	8	9
	gamb.	.06	.27	.43	.18	.06	
	arab. W				.20	.40	.40
	arab. E	.02	.36	.45	.17		
5-I		1	2	3	4		
	gamb.	.10	.61	.29			
	arab. W			.89	.11		
	arab. E	.02	.37	.59	.02		
7-I		1	2	3	4	5	6	7
	gamb.	.05	.18	.21	.31	.18	.07	
	arab. W				.29	.57	.14	
	arab. E		.04	.15	.24	.38	.13	.06
4-II		3	4	5	6	7	8	
	gamb.	.04	.21	.32	.27	.16		
	arab. W				.33	.56	.11	
	arab. E	.08	.08	.46	.29	.09		

Table 8. cont.

Seta	Species	No. branches				
6-III		1	2	3	4	5
	gamb.	.40	.36	.24		
	arab. W		.15	.46	.31	.08
	arab. E	.05	.22	.59	.09	.05
6-V		1	2	3		
	gamb.	.60	.40			
	arab. W		.92	.08		
	arab. E	.55	.45			

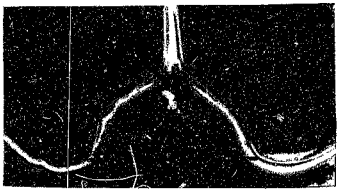
Measurements were taken of the male genital lobes (Fig. 14) described by Reid (1975a) to distinguish gambiae and arabiensis in East Africa. No usable differences were found as this character is too variable.

FIGURE 14

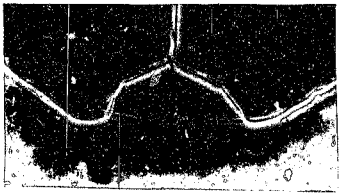
Male genital lobes of the pupae showing apparent differences between gambiae and arabiensis.

MALE PUPAL GENITAL LOBES X400

A. gambiae



A.arabiensis



4.3 Larvae

Full setal counts (Belkin, 1962) were done on at least five individuals per species. The number of branches for 344 setae per larva were recorded (Fig. 15) and setae which showed differences were chosen. Only one of each pair of setae is represented in Fig. 15. In this study the number of branches of both setae for 32 characters were recorded from 20 individuals per species. These results were assessed and more counts recorded for 11 of the characters. The results for these 11 characters are given in Table 9.

Table 9. Statistical analysis of 11 larval characters for the four species of the gambiae complex.

Seta	Species	n.	Range	Mean	S.D.
5-C	gambiae	60	13-23	17.67	2.07
	arabiensis	47	12-24	18.60	2.88
	quadriannulatus	55	12-24	18.73	2.26
	merus	59	18-28	22.00	2.27
1-P	gambiae	61	4-13	8.61	2.53
	arabiensis	51	5-15	9.53	2.48
	quadriannulatus	55	5-18	10.42	2.90
	merus	61	5-14	8.77	2.37

FIGURE 15

Line drawing of a larva showing the numbering system of Belkin (1962). The head, thorax and abdominal segments I to VI are illustrated showing dorsal setae on the left and ventral setae on the right. The terminal segments VII to X are shown in side view. (From Coetzee & Du Toit, 1979)

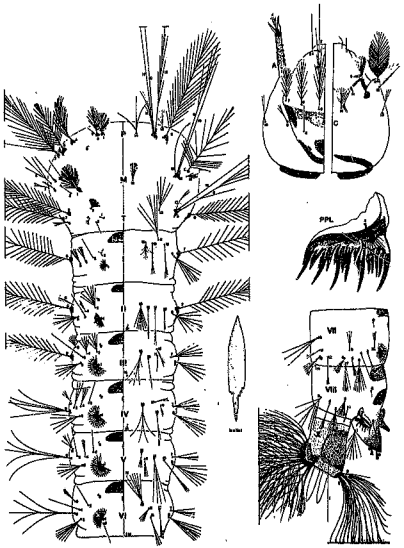

PPL
VII
VIII

Table 9. cont.

Seta	Species	n.	Range	Mean	S.D.
2-P	gambiae	60	8-18	13.35	2.26
	arabiensis	49	10-21	15.22	2.79
	quadriannulatus	58	10-21	14.36	2.43
	merus	57	8-19	14.14	2.59
1-M	gambiae	61	25-43	32.54	4.59
	arabiensis	54	23-40	32.52	4.10
	quadriannulatus	57	26-40	33.16	3.33
	merus	61	34-58	41.02	4.25
9-II	gambiae	60	7-14	10.53	1.52
	arabiensis	56	8-13	9.80	1.17
	quadriannulatus	60	7-15	10.37	1.75
	merus	62	5-10	8.00	1.20
10-II	gambiae	56	3-7	4.64	1.05
	arabiensis	49	2-5	3.45	0.65
	quadriannulatus	58	2-6	3.97	0.95
	merus	59	2-5	3.27	0.74
9-III	gambiae	57	8-12	9.40	1.22
	arabiensis	55	6-11	8.78	1.12
	quadriannulatus	61	7-12	8.98	1.22
	merus	59	4-10	7.15	1.41

Table 9. cont.

Seta	Species	n.	Range	Mean	S.D.
9-IV	gambiae	58	6-11	7.69	1.35
	arabiensis	49	5-10	7.98	1.15
	quadriannulatus	56	5-10	7.70	1.83
	merus	61	3-9	5.23	1.19
9-V	gambiae	60	5-9	7.05	1.14
	arabiensis	50	5-8	6.8	0.88
	quadriannulatus	54	5-9	6.83	0.93
	merus	61	3-8	5.16	1.23
?-VII	gambiae	48	4-8	5.85	0.82
	arabiensis	44	5-7	5.82	0.69
	quadriannulatus	44	5-9	6.75	0.94
	merus	56	3-7	4.93	0.96
3-VIII	gambiae	54	6-12	8.69	1.55
	arabiensis	41	6-13	9.71	1.69
	quadriannulatus	53	7-12	9.40	1.25
	merus	57	5-10	7.70	1.24

The Student's t-test for comparison of means was applied to all 32 characters used and these results are given in Table H, Appendix I. Nine of the 32

characters showed no significant differences between the means of any of the species ($p<0.001$). Seven of the 11 characters given in Table 9 gave t values which showed a significant difference between merus and the other three species. Two of the 11 characters (setae 2-P and 10-II) gave significant t values for the comparison of the means of gambiae and arabiensis. An. quadriannulatus showed significant differences from all the other species on seta 9-VII. The remaining character, seta 1-P_3 showed a significant t value only between a comparison of the means of gambiae and quadriannulatus.

Larval setae used by Coluzzi (1964) and Reid (1973) were examined in this study. The range and mean values are given in Table 10.

A full setal comparison between Namibian and Transvaal/Natal arabiensis was not considered as only two of the larval pelts from the Namibian sample are in suitable condition. However, the 2 selected setae given in Table 11 showed a tendency for Namibian arabiensis to be more branched than the Transvaal/Natal sample. Namibian gambiae resemble Transvaal/Natal arabiensis.

Table .10. Comparisons of larval setal branching used in this and previous studies.

	gambiae		*arabiensis*		*quadriannulatus*		*merus*		
Seta	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Source
2-C	3-14	7.89	5-16	9.91			5-15	9.68	Coluzzi (1964).
	6-14	9.17	5-13	8.84	5-13	8.14	5-13	9.47	This study.
						3.31			Ribeiro (1980).*
5-C	12-25	17.20	12-25	18.56			15-30	22.58	Coluzzi (1964).
	13-23	17.67	12-24	18.60	12-24	18.73	18-28	22.00	This study.
						13.73			Ribeiro (1980).*
6-C	13-27	18.10	11-24	19.31			16-29	22.60	Coluzzi (1964).
	14-24	18.86	16-28	20.16	16-24	20.00	18-26	21.73	This study.
						12.99			Ribeiro (1980).*
7-C	14-31	20.08	14-27	20.62			17-31	24.31	Coluzzi (1964).
	17-29	21.60	15-25	19.92	19-33	22.97	18-28	22.85	This study.
						16.29			Ribeiro (1980).*
9-C	2-7	3.45	1-5	3.06			3-6	3.96	Coluzzi (1964).
	2-5	3.26	2-4	2.63	2-7	3.71	2-6	3.85	This study.
13-C	2-8	4.39	3-8	4.68			3-8	4.81	Coluzzi (1964).
	3-6	4.30	3-6	4.11	3-7	4.57	2-7	3.98	This study.
1-P	3-13	7.41	5-16	10.74			4-15	9.13	Coluzzi (1964).
	3-10	6.16	6-13	9.40					Reid (1973).
	4-13	8.61	5-15	9.53	5-18	10.42	5-14	8.77	This study.
						4.61			Ribeiro (1980).*

*The mean values given by Ribeiro (1980) for *quadriannulatus* could not be traced in the literature quoted by him.

Table 10. cont.

	gambiae		arabiensis		quadriannulatus		merus		
Seta	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Source
2-P	7-20	12.96	6-23	14.97			10-21	14.47	Coluzzi (1964).
	8-18	13.35	10-21	15.22	10.21	14.36	8-19	14.14	This study. *
						15.21			Ribeiro (1980).
4-P	12-27	20.84	10-26	20.41			14-28	20.09	Coluzzi (1964).
	17-34	22.94	17-27	21.76	20-26	22.59	17-28	21.41	This study.
1-M	21-42	31.40	23-48	33.34			30-59	39.83	Coluzzi (1964).
	25-43	32.54	23-40	32.52	26-40	33.16	34-58	41.02	This study. *
						28.29			Ribeiro (1980).
1-I	3-13	7.69	6-20	9.64			5-12	7.88	Coluzzi (1964).
	3-9	6.44	5-10	7.05	4-10	6.70	4-9	6.66	This study. *
						4.38			Ribeiro (1980).
9-VI	5-9	6.75	3-7	4.53					Reid (1973).
	5-9	6.61	4-9	6.75	6-9	7.24	4-8	5.54	This study.
2-VII	3-8	5.24	3-9	5.20			3-5	3.92	Coluzzi (1964).
	3-7	4.87	3-6	4.14	4-6	4.83	3-6	3.90	This study. *
						3.13			Ribeiro (1980).
3-VIII	6-12	9.14	5-10	6.75					Reid (1973).
	6-12	8.69	6-13	9.71	7-12	9.40	5-10	7.70	This study.
5-VIII	3-7	4.93	3-7	4.22					Reid (1973).
	3-6	4.52	3-6	4.27	3-6	4.70	3-6	4.33	This study.
1-S	3-7	4.85	2-6	3.38					Reid (1973).
	3-7	4.47	3-8	5.29	4-8	6.00	3-7	4.53	This study.

Table 11. Setal comparisons of gambiae and arabiensis from Namibia (W) and arabiensis from the Transvaal and Natal (E).

	gambiae	arabiensis W.	arabiensis E.
Seta 12-M			
n	54	14	56
Range	1-2	1-2	1-2
Mean	1.02	1.43	1.09
S.D.	0.14	0.51	0.29
Seta 1-VII			
n	43	12	53
Range	13-20	15-20	13-19
Mean	15.77	17.75	15.70
S.D.	1.44	1.42	1.68

Four out of the seven Namibian arabiensis individuals had at least one seta 12-M bifid whereas only 3 out of 28 Transvaal/Natal arabiensis individuals had this seta branched. One gambiae individual had one seta 12-M branched while the other was simple.

The shapes of the sternal plate on abdominal segment VII (Reid 1973) were examined for all specimens. Reid (1973) reported a possible difference in shape between

gambiae and arabiensis with those individuals having the plate completely or almost divided belonging to gambiae. The amount of variation recorded in the present study was considerable and no trend could be detected in any species towards the bi-lobed state.

4.4 Eggs

The egg morphology has been extensively used in West Africa to separate melas from gambiae (Ribbands 1944, Muirhead-Thomson 1945, Bryan 1980). The eggs of melas are significantly longer and the dark opening on the dorsal surface broader than all the other species. Paterson (1962, 1964) and Kuhlow (1962) found that on size alone it was possible to distinguish egg batches of merus from those of gambiae s.s.. Coluzzi (1964) however, found it difficult to describe the differences quantitatively and suggested "Comparison on a qualitative basis seems in practice to be the most advisable method of diagnosis....".

The drying of the egg shells for scanning electron microscopy caused a tremendous amount of distortion. Unfortunately, this was noticed too late to enable measurements to be taken from a large enough sample of eggs from wild-caught females.

The measurements given below were taken from wet, unhatched eggs obtained from four colonies housed in the Botha De Meillon Insectary, South African Institute for Medical Research. The colonies were: gambiae from The Gambia, arabiensis from Zimbabwe, quadriannulatus from the Transvaal, and merus from Zululand, Natal.

Table 12. Measurements (in mm.) of colony eggs of four species of the gambiae complex.

Species	n.	Range	Mean	S.D.
LENGTH				
gambiae	26	0.50-0.55	0.52	0.02
arabiensis	50	0.48-0.55	0.50	0.02
quadriannulatus	40	0.44-0.53	0.48	0.02
merus	50	0.50-0.63	0.55	0.03
BREADTH				
gambiae	26	0.05-0.08	0.06	0.01
arabiensis	50	0.05-0.09	0.06	0.01
quadriannulatus	40	0.04-0.09	0.06	0.01
merus	50	0.06-0.10	0.08	0.01

The values for the t-test are given in Table I of Appendix I.

A comparison was made between the results in Table 12 and those published in the literature (Table 13).

Table 13. Comparison of the means of egg lengths from the present and previously published sources.

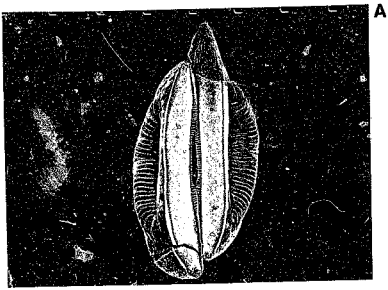
Species	Origin	n.	Mean	Reference
gamb.	Unknown colony	102	0.504	Coluzzi 1964
	Tanzania "	100	0.490	Paterson 1962
	Gambia "	26	0.52	This study
arab.	Unknown colony	62	0.499	Coluzzi 1964
	Mozambique "	100	0.487	Davidson et al 1967
	Zimbabwe "	50	0.50	This study
quad.	Swaziland	100	0.474	Davidson et al 1967
	Transvaal colony	40	0.48	This study
merus	Unknown colony	60	0.542	Coluzzi 1964
	Tanga "	100	0.575	Paterson 1962
	" "	200	0.566	Paterson 1964
	Swaziland "	50	0.546	"
	Mauritius	90	0.562	"
	"	45	0.556	"
	Zululand colony	50	0.55	This study

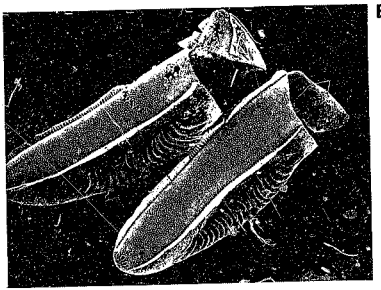
The merus eggs are significantly longer than the other three species ($p<0.001$) while quadriannulatus appears to be significantly shorter than either gambiae or arabiensis ($p<0.001$). In the present study on colony material the following percentage of individual eggs fell between the range of 0.48-0.55mm: gambiae 100%, arabiensis 100%, merus 74% and quadriannulatus 45%.

Scanning electron microscopy studies of the eggs were carried out (Fig. 16) and no differences could be detected in either the number or shape of the tubercles or of the micropyle.

FIGURE 16

Scanning electron micrographs of the eggs of: a) Anopheles arabiensis (mag. X165); b) Anopheles merus (mag. X220).


A


B

CHAPTER FIVE

DISCUSSION

5.1 Adults

In 1903 Theobald wrote about the hind leg bandings of An. costalis (= gambiae) "... in fact, I have seen fresh specimens in which it is nearly absent." Coluzzi (1964) states "Another character relates to the rings and spots of white scales on the tarsi which on the whole, are more extensive in A. merus than in A. gambiae populations examined. The ratio of the length of the white ring to length of tarsus usually gives definite discriminatory values."

Indeed, the hind leg pale band at the junction of tarsomeres 3 and 4 is a very good character for grouping gambiae/arabiensis and quadriannulatus/merus. Using the measurement 0.1mm and above, 99.6% merus (n=243) and 96.8% quadriannulatus (n=155) were grouped correctly. At 0.09mm and less, 94.0% gambiae (n=299) and 84.4% arabiensis (n=109) were grouped correctly. I did not consider the "ratio of the length of white ring to length of tarsus" to be worth measuring as the

results from the single measurement seem adequate.

White (1985) gives results of measurements of ten hind leg bands for each of the six members of the gambiae group. The mean values for the four species gambiae, arabiensis, quadriannulatus and merus correspond well with the results presented above. More data are needed for melas and bwambae before the usefulness of this character for these species can be assessed, especially in areas of sympatry with other members of the group.

Significant differences were observed between the means of some wing spot measurements and the number of coeloconic sensilla on the antennae of gambiae and arabiensis. Unfortunately, no simple combination of these characters could be found which would allow most of the females of the two species to be identified.

An. quadriannulatus and merus can be separated by plotting the total number of coeloconic sensilla against the palpal ratio, as shown in Fig. 7. Bushrod (1981) used this method to effectively separate salt-water tolerant mosquitoes (merus) from salt-water susceptible mosquitoes (gambiae/arabiensis) in Tanzania.

Some other adult characters previously reported in the

literature and not re-examined here are:

1) spermatheca size (Clarke, 1971) tested by Green (1971) and White & Muniss (1972) and found to be variable
2) wing spot ratios (Zahar et al., 1970) for which computer analysis was necessary. Discrimination was good for one locality but not for others.

These two particular character states also suffer from the drawback of having been tested initially on colony material (as do many of the reported characters noted here). Green (1971) measured the spermatheca size on wild material in Zimbabwe. He found that quadriannulatus had measurements intermediate between gambiae and arabiensis, which makes the character useless where these three species occur sympatrically.

5.2 Pupae

Table 8 in Appendix I shows a number of setae which have mean values that are significantly different. However, none of these setae, nor any combination of them, could be used with confidence to identify any of the species. The best discrimination was obtained by subtracting the sum of seta 3-III from the sum of seta 4-II. Using 7 as the cut-off value, 96.8% of the merus

sample had 7 or less branches; 80% of the quadriannulatus had 8 or more branches. The total number of these two species identified correctly was 88.5%. The level of discrimination between gambiae and arabiensis was much lower. For example, using the following combination of setae: sum 10,C plus sum 5,I minus sum 6,III, only 80% of the gambiae sample and 52.9% of the arabiensis sample could be identifi. correctly.

A comparison of the setal combinations proposed by Coluzzi (1964) (Table 6) showed some differences between his mean values and those obtained in the present study. He observed that the sums of setae 1,III + 1,IV may be of some use for the identification of gambiae and arabiensis. The mean value obtained in the present study for gambiae is somewhat higher than Coluzzi's which minimizes the taxonomic value of this character. Also, should quadriannulatus be present, any value the character may have had would be lost as this species has a mean value intermediate between the other two.

The character combinations proposed by Reid (1975a, b) to separate gambiae and arabiensis had no taxonomic value for the samples studied here (Table 7).

The interesting feature which emerged from the study

of the pupal chaetotaxy, was the geographic variation observed in arabiensis (Table 8). Unfortunately, the sample size from Namibia was very small (n=7) and more data are needed to confirm this variation. Cytogenetically and electrophoretically there appeared to be no difference between the Namibian and Transvaal populations.

5.3 Larvae

Table H in Appendix I shows numerous differences between the means of the setae examined ($p<0.001$ at a minimum of 40 degrees of freedom). An. merus showed the most differences and a number of setal combinations were tried in an effort to maximize the difference between it and the other species. These combinations proved less effective than the simple "sum of seta 9-IV". Using the sum of 9-IV as 12 or less, 87.1% of the merus sample were identified correctly; as 13 or more, 90.3% gambiae, 96.3% arabiensis and 96.7% quadriannulatus were grouped correctly. The t values given in Appendix I are rather high for this character (merus/gambiae 10.55, merus/arabiensis 12.26, merus/quadriannulatus 8.72) but the practical discrimination of individual merus is nonetheless not very good (only 87.1%). No attempt

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was made to find setal combinations to discriminate gambiae and arabiensis where the highest t value is only 6.88.

A comparison of setae used by Coluzzi (1964) and Reid (1973) with the present samples shows some differences in mean values and in some cases this affects the taxonomic value of the character. Examination of the t values in Table H, Appendix I, shows 6 out of the 16 characters used by Coluzzi and Reid to have very little or no statistical significance. The mean number of branches on setae 5-C and 1-M show the greatest statistical difference between merus and the other species and of these two, seta 1-M is the best for separating merus from the others. Where the sum of the branches of seta 1-M was 76 or more, 83.9% merus were identified correctly; a sum of 75 or less groups 83.9% gambiae, 96.4% arabiensis and 90.3% quadriannulatus. This character is not as good as seta 9-IV mentioned above.

Prothoracic seta 1 (1-P) has been used extensively since first proposed by Coluzzi (1964). Coluzzi found that in colonies of gambiae and arabiensis originating from Pala, Burkina Faso (Upper Volta), only 10% of his sample fell in the overlap range of 8-11 branches. In othe cases, though, discrimination was not as good. Paterson (1968) tested this character at Chirundu,

Zambia, where gambiae, arabiensis and quadriannulatus occurred sympatrically and found it to be of no value. Green (1971) studied a sample of quadriannulatus from Chiredzi/Lundi, Zimbabwe, and found that 52% of his sample fell in the range 8-11 branches. He concludes that this character cannot be used where quadriannulatus occurs sympatrically with either of the other freshwater species. The present study shows that virtually no value can be attached to this character in southern Africa, with only a slight significance (t=3.60) between the means of gambiae and quadriannulatus, and no differences between the means of any of the others.

Two possible explanations for the differences seen between Coluzzi's and Reid's results and my own are: a) their extensive or exclusive use of colony bred material, and b) possible localized geographic variation.

The means for quadriannulatus in Table 10 taken from Ribeiro (1980) are vastly different from those obtained in the present study. In the text, Ribeiro lists his source of data but the figures that he quotes for quadriannulatus cannot be found in the publications cited (Coluzzi 1964, Davidson et al. 1967, Ismail & Hammoud 1968, White 1973, 1974, Reid 1975a, b, Ribeiro et al. 1979). At the same time,

White (1973) gives coefficients of difference between quadriannulatus and "species D" and refers to himself "White, 1973". The raw data for quadriannulatus was never published (White, pers.comm.) but White (1973) does state that "... as regards the larva, species C resembles species B most closely and differs only a little from species A." The differences between Ribeiro's (1980) figures and my own are enormous (eg. seta 2-C has a mean of 3.31 according to Ribeiro and 8.14 from my data). His data should serve to identify quadriannulatus with little difficulty. In fact, most of the mean values he gives are startlingly similar to mean values for the West African salt-water breeder melas (Coluzzi, 1964). In fact, what Ribeiro did was to use White's (1973) coefficients of difference and work out the mean values for quadriannulatus with the assumption that the standard deviations of quadriannulatus are the same as those of his "subspecies" quadriannulatus davidsoni (Ribeiro, pers. comm.). He considers this to be a reasonable assumption. It is, however, unacceptable. By definition (Mayr, 1969, p. 41), a subspecies must differ taxonomically from other populations of the species. Thus, q. davidsoni must be different from quadriannulatus, and the use of the same standard deviations is not, in fact, reasonable. Also, it is unacceptable in statistical analyses to assume that the standard deviations of two samples will be the same. The data given for quadriannulatus

by Ribeiro (1980) must, therefore, be disregarded.

Geographic variation was again noted between arabiensis populations from Namibia and Transvaal/Natal. Two populations of gambiae (Brazzaville, West Africa and Grand Comoros island off East Africa) were examined for geographical variation and two setae, 11-C and 1-M, differed to some extent. Seta 1-M showed the most difference and, in fact, the 16.1% of the sample which overlapped with merus for this character (see above) all came from Grand Comoros.

Unfortunately, once again no morphological character was found to distinguish individual larvae of each species.

5.4 Eggs

The measurements from this study given in Tables 12 and 13 can only serve as an indication of differences between the species. Throughout this study I have constantly avoided using colony material and it is unfortunate that the eggs from wild females could not be measured (see 4.4). My results of quadriannulatus egg lengths indicate a difference between it and gambiae and arabiensis. The mean length value of 0.48mm is very similar to that (0.474mm) published by

Davidson et al. (1967). They, however, state that "... it thus seems impossible to distinguish the three freshwater species from the lengths of their eggs." If statistical analysis was carried out to test the differences between the means, Davidson et al. do not report this. The finding that merus eggs are significantly longer than the three freshwater breeders is in agreement with previously published data (Table 13).

5.5 Museum collections

Specimens were examined from four museum collections: a) British Museum (Natural History); b) London School of Hygiene and Tropical Medicine; c) Smithsonian Institution; d) Biosystematics Research Institute, Ottawa. There are drawbacks to all these museum specimens in one way or another. Many of the labels lack information on method of identification, or date back to before the gambiae complex was defined, or are labelled "gambiae group". Many of the specimens originate from laboratory colonies. Some specimens labelled as chromosomally identified still had large blood-filled abdomens which makes it doubtful that usable chromosome preparations were actually obtained from these specimens.

However, leg-banding measurements were taken and these are tabulated in Appendix II, together with the predicted group each specimen should belong to based on the leg-banding criteria above. Despite the reservations about the identification of most of the specimens, the majority conform surprisingly well to the leg-banding groups. I do, however, question the identification of 7 specimens of quadriannulatus (in the BMNH) from Chirundu, Zambia, collected in houses. The leg bandings indicate that these may have been misidentified.

5.6 Discriminant Function Analysis

A computer multivariate discriminant function analysis (SAS software) was used in an attempt to maximize the separation of the four species. A summary of the material examined and the number of characters which showed significant differences are given in Table 14. Thirteen of these characters were chosen for the computer analysis. They are: the hind leg banding patterns; the number of coeloconic sensilla on segments 5, 6, 9 and the total number; the palp index; the sum of pupal setae 10-C, 5-I, 4-II, and 6-III; the sum of larval setae 2-P and 10-II; the egg length. A total of 100 specimens were used.

Table 14. A summary of material examined and characters showing significant differences.

No. Adults examined	806	
No. Pupae examined	160	
No. Setae on each pupa	344	
Total setae examined	11 960	11 960
No. Larvae examined	120	
No. Setae on each larva	344	
Total setae examined	14 640	14 640
No. Eggs examined	166	
		26 600

Characters showing significant differences

Adults	18
Pupae	10
Larvae	23
Eggs	1

Using a stepwise method and running all four groups at once, 97% total discrimination was achieved (Figs 17 and 18). One gambiae individual was misplaced in the arabiensis group, and two arabiensis individuals were misplaced, one each in the gambiae and quadriannulatus groups.

The following key was devised based on the characters found to have the highest discriminating value by the computer analysis.

1. Pale band at the joint of hind tarsomeres 3 and 4, 0.1mm or more.....2
- This pale band 0.09mm or less.....3
2. Palpal ratio of 0.85 or higher.....merus
- This ratio 0.84 or lower.....quadriannulatus
3. The sum of coeloconic sensilla on flagellar segments 5 + 6 + 9 of both antennae is 13 or more.....arabiensis
- This sum is 12 or less.....gambiae

This simple key identifies 95% merus, 89% quadriannulatus, 78% arabiensis and 76% of the gambiae females used in this study. The probability of correct identification is increased if a minimum of three progeny of a wild female are used and an average measurement or count used for the key. The above percentages increase to 100, 100, 87.5 and 94 respectively.

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FIGURE 17

Computer printout of discriminant function analysis of four members of the gambiae group, with merus clearly separated on the right. 1=gambiae; 2=arabiensis; 3=quadriannulatus; 4=merus.

SAS

PLOT OF CANONICAL VARIATES 1 AND 2 SYMBOL IS VALUE OF GROUP

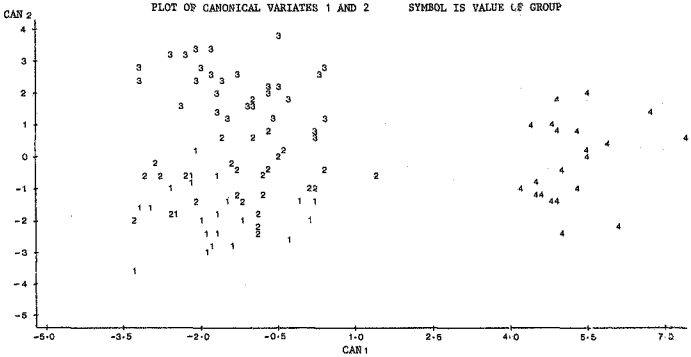
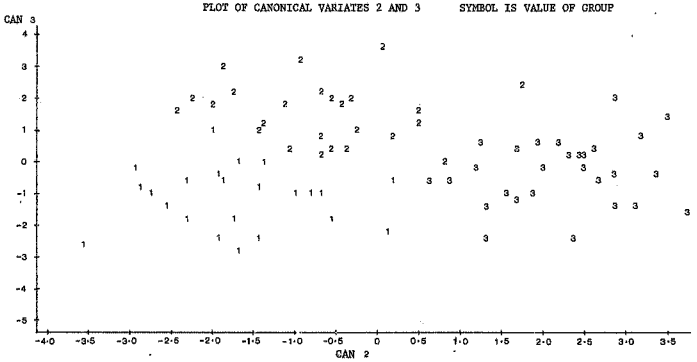


FIGURE 18

Computer printout of discriminant function analysis of the three freshwater breeding members of the gambiae group. 1=gambiae; 2=arabiensis; 3=quadriannulatus.

SAS

PLOT OF CANONICAL VARIATES 2 AND 3 SYMBOL IS VALUE OF GROUP



5.7 General

An attempt was made to examine as many of the reported differential characters as possible. Some, however, were disregarded as they had already been discredited in the literature and there seemed little point in pursuing them. For example, measurements of the spermathecae were not done as Green (1971) had shown, using wild material, that Clarke's (1971) character had no practical value in the field where quadriannulatus may occur.

The exclusive use of F-1 progeny from wild-caught females had serious limitations. Wild females were collected from numerous localities, however, not all survived, nor did they all lay eggs. Some egg batches obtained were not successfully bred out in the laboratory. All in all, my sample size was severely restricted by using F-1 progeny, as opposed to colony material. However, the advantage of knowing that the samples most probably resemble wild material very closely, compensates for lower numbers. Obviously more data are needed and from many more localities to show whether the morphological characters reported here are consistent within species and applicable in other areas.

CHAPTER SIX

CONCLUSION

It can be said that there are two distinct kinds of human endeavour in the field of evolutionary biology: a) the science of classification, and b) the study of gene exchange and its consequences. Once a complex of species has been sorted out genetically, we can then fit them into our system of classification. Two distinct activities in two distinct fields of endeavour with no judgements about which is more important. They have equal status. This thesis deals essentially with the science of classification and the identification of a group of cryptic species.

The identification of vector species is of fundamental importance for without it we cannot study the biology of the individual species, work out the epidemiology of a disease or study the spread of resistance; nor can we begin to formulate ways of controlling them. One of the major flaws of the experimental malaria control project in the Garki district of Nigeria (Molineaux & Gramiccia, 1980) was the initial lack of identification of the vectors. The reasons for the

failure of the spraying programme would have been known much sooner if the workers had been aware of the species with which they were dealing.

At present, chromosomal analysis is the most precise and quickest means of identifying individual members of the gambiae group. A study such as that reported by Shelley (1973), however, does little to instill confidence in the technique. The identification of quadriannulatus by "ravelled" polytene chromosomes (Shelley, 1973) is totally inadequate and factually incorrect. Preparations of polytene chromosomes from quadriannulatus can be just as good as those obtained from the other members of the complex. The publication of papers like this is counter-productive in the fight against malaria.

Chromosomal identification does, however, have limitations. Morphologically very distinct species can have homosequential chromosomes, such as Drosophila silvestris and heteroneura (Craddock, 1974). Similarly, morphologically similar species, such as Anopheles funestus and ?aruni (De Meillon et al., 1977) may have homosequential chromosomes (Green & Hunt, 1980). These latter two species were recognised from cross-mating studies. True cryptic species with homosequential chromosomes and no evidence of hybrid sterility would not be recognised. This is probably

the case in West Africa where Bryan et al. (1982) demonstrated linkage disequilibrium and heterozygote deficiencies of polymorphic inversions in populations of gambiae from The Gambia. Coluzzi et al. (1985) suggest "incipient speciation" for two populations of gambiae from Mali which show a complete lack of heterozygotes between certain chromosomal inversions. These West African populations show no signs of hybrid sterility nor chromosomal asynapsis when crossed in the laboratory.

The electrophoretic separation of enzymes can confirm the lack of gene flow evident between sympatric populations. However, electromorph similarity does not necessarily mean a single gene pool exists and many instances are known where chromosomally distinct species have identical electromorph frequencies (see Futuyma, 1979, p419, Lambert & Paterson, 1982). The use of electrophoresis for identifying individual members of the gambiae complex is less precise than chromosomes, but this method is quite adequate for population studies. Once electromorph frequencies have been established for a species in a given area, a large percentage of the unknowns can be identified with confidence (Miles, 1979). Individuals possessing rare electromorphs, however, have to be disregarded unless correlated with chromosomal identification as was done with the gambiae samples from Brazzaville and

Grand Comoros.

Morphological identification of members of the gambiae complex is the least efficient method available. Moreover, this study has shown that previous morphological studies of the group based on colony material cannot be applied in the field, at least, to southern African material.

The description of a subspecies of quadriannulatus (Ribeiro et al., 1979) based purely on morphological criteria is not acceptable in the light of present day knowledge. As shown by Cambournac et al. (1982), quadriannulatus davidsoni from the Cape Verde islands is chromosomally identical to arabiensis from the nearby mainland of Senegal. Morphological geographic variation is not a valid criterion for the naming of a subspecies within a group of species which are defined purely on genetical criteria.

Morphological variation in different geographic populations does pose an interesting question: is the variation seen as an indication of different species, or merely environmentally induced variation? Studies by Coluzzi (1964) and Reid (1973, 1975a, b) indicate a difference between West African and East African populations of gambiae and arabiensis. The present study shows differences between Namibian and

Transvaal/Natal arabiensis and Brazzaville and Grand Comoros gambiae. Studies on other Anophelinae from northern Namibia indicate that the species found there are not found in either the Transvaal or Natal. An. pharoensis from Namibia is not the same as that in Natal (Miles et al., 1983). An. "ziemanni" (actually An. namibiensis Coetzee 1984) was also shown to be different from ziemanni in South Africa. Species occurring in Namibia (eg. wellcomei) are not found in South Africa (Gillies & De Meillon, 1968). Circumstantial and very limited morphological evidence indicate that arabiensis in Namibia may be a different species from that collected in south-eastern Africa. Whether the morphological differences between the Brazzaville and Comoros gambiae indicate the same thing for this species is much more speculative. There is absolutely no cytogenetical or electrophoretic evidence to support these speculations.

Phylogenies of the gambiae group have been based on chromosomal inversions (Coluzzi & Sabatini 1969, Coluzzi et al. 1979) and morphological differences (Ribeiro, 1980) (see Appendix III). The current method of approaching a phylogenetic relationship based on chromosome inversions requires an out-group comparison (Carson 1970, Green 1982, Green et al. 1985b). This has not been done for the gambiae complex (Coluzzi et al., 1979). Relationships based on morphology

(Ribeiro, 1980) are suspect due to the nature of the data used. Not only has colony material been shown to be inadequate, but the data used by Ribeiro for quadriannulatus was obtained by a method which is unacceptable statistically. Furthermore, the use of quadriannulatus davidsoni is invalid as this has been shown to be arabiensis (Cambournac et al., 1982). All conclusions drawn by Ribeiro are therefore also invalid.

The problems of identification of the gambiae complex are by no means unique within the Anophelinae. Examination of the literature shows that great confusion reigns over the question of the An. balabacensis complex in South East Asia. Some members, or "strains", of this group are efficient vectors of malaria and some are not (Reid, 1968). This is a strong indication that one is dealing with more than one species. Hybridization studies done by Kanda et al. (1985) on An. takasagoensis Peyton & Harrison 1980 and five "strains" of balabacensis showed considerable male sterility between crosses of strains within balabacensis. One "strain", however, yielded fertile male and female offspring when crossed to takasagoensis. This, according to Kanda et al. (1985), indicates that takasagoensis is a variation of balabacensis and not a separate species. These hybridization studies were performed on laboratory

colonies. Also reported in the literature, but ignored by Kanda et al. (1985), is the record of a species An. dirus Peyton & Harrison 1979 . This name has been allocated to a "strain" of balabacensis and Kanda et al. do not indicate which of their strains is dirus. An added complication is that "dirus" has been shown to consist of two species (Hii, 1985). All the cross-mating and chromosomal studies, of which I have mentioned very few, have been conducted on colony material. Some of these colonies are known to be hybrids (Green, pers.comm.).

Speculation regarding allopatric populations should take into account current species concepts and learn from examples like the gambiae complex. Unlike Kanda, Hii (1985) comes closest to these ideals and the conclusions he draws are compatible with the data presented, i.e., balabacensis consists of several different species, including dirus. All the above studies mentioned were based either on cross-mating or chromosomes or both. No satisfactory answer to the balabacensis question will be obtained until workers on the group sample directly from nature and correlate chromosomes with morphology.

A sad aspect of all the studies mentioned here is that very few species identifications of field specimens can be correlated with other aspects of, say,

behaviour or morphology. Ideally, each individual wild female should be studied for a) chromosomal identification, b) blood-meal analysis, c) sporozoite infections, d) morphology, and e) electrophoresis. This is not as impossible as it sounds and a full description of the necessary techniques has been presented by Hunt & Coetzee (1986a). By correlating all this information for individual animals, an enormous body of data will become available and numerous questions can be answered for whatever group one is analysing. It might even, in the future, become possible to identify individuals using DNA probes and this would add another dimension to the data set. Possibly questions like "Is An. arabiensis in Namibia the same species as that in Natal?" may be answered. Perhaps applying all available techniques to the West African populations would explain some of the results obtained by Bryan et al (1982) and Coluzzi et al. (1985). However, it is important to remember that the genetical characters used to identify the species are *only markers of the lack of gene flow (Miles, 1981).* It is not chromosome inversion or electromorph differences which delimit the field for gene recombination. It is behaviour, i.e., positive assortative mating which defines the limits of the gene pool.

With all these sophisticated genetical techniques to

identify individuals, it may be asked: what is the function of classical taxonomy? The role of taxonomy when applied to anopheline mosquitoes has become almost obsolete. That is, when applied in the traditional manner which is the description of absolute morphological differences between species (sometimes based on a single specimen!). This is no longer applicable for groups of cryptic species identified by genetical markers, such as the gambiae complex. No absolute morphological differences were recorded for the gambiae complex before I started, and this study has not come up with any either! Morphological studies on anopheline species must be based on the progeny of identified wild females. This enables one to assess the variation within populations, while minimizing the risk of dealing with mixtures of species.

Having obtained an adequate correlated data base for a population or populations, and assuming that genetical methods have revealed new species within a single taxon, it should then be simple to test morphological characters for discrimination of genetical species. Furthermore, one should be able to establish which, if any, of the previously described and named synonyms of the taxon might be assigned to the new genetical species. This approach was followed by Lambert & Coetzee (1982) in their study on the An. marshallii

group. They used multivariate discriminant function analysis to separate the adults of the marshallii group of species. One of the major benefits resulting from the computer analysis was that when the type specimens of marshallii and its synonyms (i.e., An. pitchfordi, An. transvaalensis and An. pseudocostalis) were entered into the programme as unknowns, it was possible to predict with 95% confidence which genetical group they belonged to. An. transvaalensis was shown to be the same as marshallii (= species B of the group), while pitchfordi and pseudocostalis were not grouped by the computer. Based on this analysis, Lambert & Coetzee concluded that the other three members of the marshallii group (species A, C and E) were new species and named two of them (species A = An. letabensis, C = An. hughi). Subsequently, more material of species E has been obtained and this has been described as An. kosiensis (Coetzee et al., 1986).

A combination of all available techniques and their logical application is now essential for the understanding of the systematics of insect vectors of disease pathogens. The obvious limitations inherent in the current identification techniques may be minimized if a combined approach is used. Ultimately, the characters used have to be related to the behavioural characters which determine the limits of gene exchange in nature.

APPENDIX I.

STATISTICAL ANALYSIS

Table A. Wing spot measurements (1 unit = 0.04mm).

Spot	Species	n.	Range	Mean	S.D.
1	gambiae	19	7-11	8.84	1.07
	arabiensis	90	7-13	9.62	1.31
	quadriannulatus	100	6-12	9.27	1.40
	merus	73	6-14	9.18	1.80
2	gambiae	19	2-6	3.53	0.91
	arabiensis	90	3-9	4.26	0.92
	quadriannulatus	101	2-7	4.05	0.97
	merus	73	3-6	4.53	0.77
3	gambiae	19	14-20	17.11	1.56
	arabiensis	90	11-22	17.40	2.58
	quadriannulatus	101	10-20	15.67	2.27
	merus	73	11-25	16.34	3.31
4	gambiae	19	7-11	8.89	1.10
	arabiensis	90	5-13	8.53	1.46
	quadriannulatus	101	7-15	9.37	1.36
	merus	73	6-17	9.06	1.54

Table A. cont.

Spot	Species	n.	Range	Mean	S.D.
5	gambiae	19	8-14	11.84	1.61
	arabiensis	90	6-15	11.21	1.66
	quadriannulatus	101	8-16	11.32	1.59
	merus	73	5-20	11.59	2.92
6	gambiae	19	5-8	6.79	0.71
	arabiensis	90	4-12	7.96	1.39
	quadriannulatus	101	4-10	7.07	1.18
	merus	72	5-10	7.51	1.10
7	gambiae	19	4-5	4.47	0.51
	arabiensis	90	3-7	5.09	0.92
	quadriannulatus	100	3-7	5.04	0.95
	merus	69	3-10	5.80	1.31
8	gambiae	19	2-6	4.00	1.16
	arabiensis	90	1-10	4.02	1.28
	quadriannulatus	100	2-10	4.46	1.31
	merus	69	0-13	4.13	2.16

Combined spots 2+7.

Species	n.	Range	Mean	S.D.	t	p<0.001
quad.	37	6-13	9.11	1.51		
merus	26	9-14	10.62	1.42	4.015	

Table B. Students t-test of wing spots ($p<0.01$).

Species pairs	Spot 1	Spot 2	Spot 3	Spot 4
quad/gamb	1.27	2.16	2.643	1.449
quad/merus	0.37	3.5	1.582	1.404
quad/arab	1.773	1.528	4.925	4.122
merus/gamb	0.787	4.882	0.982	0.452
merus/arab	1.804	2.008	2.295	2.256
gamb/arab	2.425	3.155	0.471	1.017

	Spot 5	Spot 6	Spot 7	Spot 8
quad/gamb	1.308	1.001	2.396	1.347
quad/merus	0.783	2.49	4.361	1.232
quad/arab	0.468	4.791	0.367	2.333
merus/gamb	0.358	2.7	4.312	0.252
merus/arab	1.043	2.242	4.008	0.4
gamb/arab	1.512	3.563	2.84	0.063

Table C. Hind leg pale band measurements (in mm.) at joint of tarsomeres 3/4.

Species	n.	Range	Mean	S.D.
gambiae	299	0.04-0.14	0.07	0.02
arabiensis	109	0.04-0.13	0.08	0.02
quadriannulatus	155	0.08-0.20	0.14	0.02
merus	243	0.08-0.20	0.14	0.02

Table D. Students t-test of hind leg bands ($p<0.001$).

Species pairs	t
quad/gamb	27.51
quad/merus	1.58
quad/arab	19.60
merus/gamb	17.86
merus/arab	20.41
gamb/arab	2.99

Table E. Students t-test of coeloconic sensilla ($p<0.001$).

Flagellum segment	quad/ gamb	quad/ merus	quad/ arab	merus/ gamb	merus/ arab	gamb/ arab
1	1.69	7.78	0.20	10.69	7.86	2.00
2	0.69	7.68	3.21	10.26	4.92	4.90
3	1.64	8.14	0.69	11.87	7.21	2.49
4	2.80	10.16	1.76	13.65	8.02	4.53
5	4.31	5.37	1.33	11.54	6.43	4.90
6	5.26	9.78	1.48	12.64	5.20	6.11
7	4.14	3.55	1.74	8.55	5.37	1.16
8	0.98	0.07	2.48	1.99	2.51	3.98
9	3.05	0.11	1.61	2.97	1.85	4.48
Total	5.01	10.22	1.40	17.67	8.73	6.75
1+2	1.43	8.97	1.89	12.41	7.63	3.93

Table F. Students t-test of palp ratios ($p<0.001$).

Species pairs	t
quad/gamb	3.64
quad/merus	10.59
quad/arab	0.42
merus/gamb	11.23
merus/arab	9.58
gamb/arab	2.72

Table G. Students t-test of pupal setae ($p<0.001$).

Data	quad/ gamb	quad/ merus	quad/ arab	merus/ gamb	merus/ arab	gamb/ arab
10-C	0.96	3.22	2.28	4.68	0.63	3.57
4-I	2.69	5.89	2.84	3.52	3.29	0.24
5-I	5.33	8.87	1.35	3.00	6.82	3.82
7-I	2.65	2.86	1.86	5.37	4.70	1.08
4-II	5.19	9.33	4.44	3.58	4.82	0.87
6-III	3.36	1.95	1.20	0.79	2.97	4.69
6-IV	0.25	0.93	1.76	0.72	0.85	1.61
3-V	2.09	2.67	4.04	0.54	1.70	2.43
6-V	0.89	3.71	0.49	4.84	4.26	0.40
3-VI	0.23	1.69	0.25	1.79	2.19	0.56
7-VII	2.89	1.71	4.26	2.86	4.22	1.49
3-III	0.60	4.79	1.10	4.35	4.72	1.74

Table H. Students t-test of larval setae ($p<0.001$).

Seta	quad/ gamb	quad/ merus	quad/ arab	merus/ gamb	merus/ arab	gamb/ arab
2-C	1.85	2.53	1.19	0.52	1.05	0.52
5-C	2.63	7.71	0.26	10.87	6.80	1.94
6-C	2.44	3.70	0.33	5.71	3.01	2.47
7-C	1.89	0.20	5.08	2.06	5.99	2.72
9-C	2.01	0.59	5.05	3.01	6.67	3.79
11-C	1.16	1.91	2.40	0.45	0.89	1.17
13-C	1.33	2.61	2.23	1.59	0.63	1.08
1-P	3.60	3.37	1.69	0.37	1.65	1.94
2-P	2.35	0.47	1.71	1.76	2.08	3.88
4-P	0.37	2.48	1.31	2.02	0.59	1.13
1-M	0.83	11.12	0.90	10.58	10.88	2.75
13-M	3.22	5.04	5.93	0.94	1.85	2.29
1-I	0.96	0.14	1.23	0.79	1.34	2.23
4-I	3.69	8.12	1.96	5.15	6.11	1.59
9-I	0.33	10.99	2.75	8.96	7.52	2.01
13-I	1.60	1.86	1.15	0.08	0.61	0.47
5-II	0.16	3.38	1.19	2.74	1.68	0.92
9-II	0.56	8.75	2.03	10.22	8.26	2.88
10-II	3.60	4.40	3.21	8.13	1.32	4.88
13-II	3.27	2.05	4.94	1.32	2.92	1.48
2-III	1.95	4.15	1.16	2.28	2.64	0.63
6-III	1.66	0.32	1.52	1.56	1.38	0.23
7-III	2.53	5.39	2.84	3.29	2.75	0.46
9-III	1.87	7.62	0.93	9.17	6.80	2.81

Table H. cont.

9-IV	2.25	8.72	0.94	10.55	12.26	1.18
9-V	1.11	8.15	0.19	8.75	7.90	1.27
9-VI	2.89	8.01	1.97	4.71	4.79	0.52
2-VII	0.22	5.94	4.19	4.61	1.30	3.25
9-VII	4.86	9.39	5.29	5.12	5.07	0.23
3-VIII	2.61	7.15	1.03	3.70	6.78	3.06
5-VIII	1.20	2.49	2.90	1.09	0.39	1.46
1-S	6.61	6.57	2.33	0.26	2.77	2.85

Table I. Students t-test of egg measurements ($p<0.001$)

Species pairs	Length	Breadth
quad/gamb	8.15	0.05
quad/merus	14.63	9.91
quad/arab	5.73	0.49
merus/gamb	6.08	7.16
merus/arab	12.38	8.68
gamb/arab	0.64	0.51

APPENDIX II

HIND LEG BANDING MEASUREMENTS OF MUSEUM COLLECTIONS OF THE Anopheles gambiae GROUP.

British Museum (Natural History)

Species	Locality	Collection	Det.*	n.	Range (mm)	Leg band group
quadri-annulatus	Mazoe Valley, Zimb.	Net trap	?	13	0.1-0.18	quadriannulatus/merus
	Chirundu, Zambia	In houses	?	7	0.06-0.09	gambiae/arabiensis
merus	Mauritius	Colony	Ross Inst.	3	0.1-0.14	quadriannulatus/merus
	Pemba, Tanzania	Cattle trap	Gillies	14	0.12-0.16	quadriannulatus/merus
gambiae	The Gambia	Holotype	Burdett	1	0.03	gambiae/arabiensis
	Nigeria	Colony	Ross Inst.	13	0.02-0.08	gambiae/arabiensis
	Liberia	Colony	Ross Inst	3	0.02-0.05	gambiae/arabiensis
	Kenya	Colony	Ross Inst.	2	0.11-0.12	quadriannulatus/merus
melas	Freetown, Sierra Leone	Bred from eggs	Muirhead-Thomson	9	0.02-0.06	
	Keneba, Gambia	Colony	Ross Inst.	2	0.02-0.04	
	No data			4	0.02-0.05	
bwambae	Uganda	No data		7	0.08-0.14	

London School of Hygiene and Tropical Medicine

quadri-annulatus	Lundi, Zimbabwe	No data		26	0.11-0.17	quadriannulatus/merus
	Ethiopia	No data	Ross Inst.	6	0.08-0.11	gambiae/arabiensis
	Ethiopia	Cattle shed	?	8	0.06-0.10	gambiae/arabiensis
merus	No data			12	0.08-0.18	quadriannulatus/merus

Smithsonian Institution

Species	Locality	Collection	Det.*	n.	Range (mm)	Leg band group
merus	Tanganyika	?	Coluzzi	2	0.13-0.16	quadriannulatus/merus
melas	Freetown, Sierra Leone	Salinity tests	Ribbands	3	0.03-0.05	
	Liberia	No data		17	0.05-0.13	
arabiensis	Nigeria	?	Coluzzi	3	0.03-0.06	gambiae/arabiensis
gambiae	Gambia	No data		1	0.06	gambiae/arabiensis
	Kenya	?	Coluzzi	2	0.06-0.10	gambiae/arabiensis
quadri-annulatus	South Africa	No data		2	0.12-0.13	quadriannulatus/merus
bwambae	Uganda	Biting man	?	1	0.14	

Biosystematics Research Institute, Ottawa

?	Dar es Salaam	Biting man	?	7	0.1-0.2	quadriannulatus/merus

*Det. = Determined or identified by.

APPENDIX III

PHYLOGENETICS OF THE ANOPHELES GAMBIAE GROUP.

Phylogenetic relationships based on X chromosome inversions for the members of the gambiae group were first proposed by Coluzzi & Sabatini (1969). A later phylogenetic tree for the group (Coluzzi et al., 1979) based on all known inversions within the complex favoured quadriannulatus as the ancestral species. However, no outgroup comparisons were made (Carson 1970, Green 1982, Green et al. 1985b). An. quadriannulatus was chosen as the ancestor a) because of its "relict" distribution, and b) for its preference for animal hosts (as opposed to mixed animal/man biting behaviour). In a more recent publication, White (1985) postulates that bwambae is a recently evolved member of the group as the Semliki forest is supposedly only 9000 years old.

Using the technique developed by Vrba (1979) and followed by Green (1982), the chromosomal inversions are schematically presented in Fig. 19, with quadriannulatus as the standard arrangement in A and gambiae as the standard in B. However, as no outgroup has been included, the black squares only indicate inversion sequences unique to each species and do not

FIGURE 19.

Shared and unique inversions of members of the gambiae complex; a) using quadriannulatus as the standard arrangement, and b) with gambiae as the standard but not showing the inversion polymorphisms.

X a
bcd
e
f
2 a
ah
b
bc
be
bf
bk
br
d
g
i
j
l
m
n
op
q
s
t
u
3 a
b
c
d
4 a
b
c
d
e
5 a
quadriannulatus
bwambae
melas
merus
gambiae
arabiensis

A

Xa
Xabcd
2m
2op
3a
5a
quadriannulatus
bwambae
melas
gambiae
merus
arabiensis

B

confer derived or ancestral status on the characters used. Half squares indicate polymorphic inversions. Where black squares are shared by two or more species, one can postulate that they shared a recent common ancestor. Fig. 19b depicts the fixed inversions unique to each species or groups of species using gambiae as the standard. All it is possible to say from these data is that melas, bwambae and quadriannulatus may share a common ancestor, gambiae and merus probably do too, and merus and arabiensis might have done. The fact that merus and arabiensis share the inversion 3a and that this inversion is polymorphic in gambiae may indicate that the common ancestor of these three species had 3a as a polymorphism.

The phylogenetic tree in Fig. 20 postulates that inversion polymorphisms have been lost in many populations as I have taken into account the need for an inversion sequence to have arisen initially as a heterozygote. Obviously, everything below the species names is speculation and in fact many more extinct populations may conceivably have existed. However, without an outgroup, little weight can be attached to these diagrams.

The speculation by White (1974) and Coluzzi et al. (1979) that quadriannulatus is the ancestral form

FIGURE 20

A postulated phylogenetic tree. The "extinct" populations represent unique inversions which presumably arose as heterozygotes before becoming fixed in one species or the other.

melas
bwambae
quadriannulatus
gambiae
merus
arabiensis
Xa
5a
2m
Xa
5a
Xa
5a
2m/+
Xa
5a/+
3a/+
Xa/+
3a/+
Xa/+b/+
3a/+
2o/+b/+
3a/+
3a
Xab, c/+
3a/+
3a
2op
3a
2o, p/+
3a
Xabc, d/+
3a
Xabcd

cannot be supported by the chromosomal data. Coluzzi et al.'s (1979) assertion that the chromosomes of quadriannulatus are "central" in the group does not stand up to critical evaluation. In fact, quadriannulatus differs from gambiae by a single fixed inversion on the X chromosome. One can postulate that the longer a species has been in existence the more chance it has had to accumulate inversion rearrangements. In that case, gambiae is a better candidate for the ancestral form than quadriannulatus as it has eight polymorphic inversions while quadriannulatus only has two. Under the same premise, arabiensis with 16 polymorphic inversions would be the ancestral species, while merus having none would be considered to be the most recently evolved. The use of quadriannulatus as the standard chromosome arrangement (Coluzzi et al., 1979) is a purely arbitary decision and any other member of the group can serve just as well, as is shown in Fig. 19.

Another reason given by Coluzzi et al. (1979) and White (1974) for the primitiveness of quadriannulatus is its tolerance for temperate climates. This may be so in Ethiopia, but in Zimbabwe and South Africa quadriannulatus' preferred habitat is in the hot dry lowveld regions (Hunt pers. comm. and personal observations), a preference shared by many arabiensis populations in Africa. The animal biting behaviour of

quadriannulatus may indeed be ancestral but this is also shared by other members of the group. One could just as well argue that the catholic behaviour of merus or arabiensis (bites cattle or man) is more suited for the ancestral form than the specialized behaviour of either quadriannulatus (cattle biting) or gambiae (man biting).

According to White (1985) "Since the Gamblian pluvial peak, about 9000 years ago, African lake levels have receded (Bishop, 1971) and the habitat of An. bwambae has formed in the Semliki valley." Thus, bwambae cannot have speciated more than 9000 years ago. As the distribution of bwambae is restricted to the Semliki valley, Uganda (as far as is known), speculation on the maximum age for the species based on the geological history of the area is valid. To suggest a maximum age for the species, one must consider the age of rifting, the criteria needed to produce the haline springs and the age of emergence of dry land between the two lakes. The Lake Albert - Semliki - Lake Edward Rift valley (sometimes referred to as the Albertine Rift) has been active for several million years in response to crustal thinning in this part of Africa. The last major rejuvenation of the rift boundary faults and production of the present topography was during the mid-Pleistocene (Bishop, 1965). Downfaulting of the graben, which continues to the

present day, will have ensured high heat production in the vicinity of the boundary faults. This, combined with meteoric water input (Arad & Morton, 1969) produces hot springs. A source of meteoric water will have been available since the lakes came into being which may have been as long ago as 15 million years - the age of the Oligocene, lower Miocene PIII drainage surface (Gautier, 1965). Hence, the most important parameter to determine a maximum age for bwambae is the age of emergence of dry land between lakes Edward and Albert and the formation of the Semliki valley.

Given that the hot springs are situated along the escarpment edge, the environment of the species would have been destroyed only when the graben was entirely water-filled. The emergence of the Semliki valley occurred before the Gamblian pluvial but later than the Kamasian pluvial, within the Upper Pleistocene (Cahen 1954, Bishop 1971). Since emergence, there have been periodic recessions of the Semliki River leaving terraces 6m, 12m and 40m above the river level during the Nakurian, Makalian and Gamblian periods respectively. The Fauresmith stone industry, examples of which are found on the 40m terrace, has been dated at approximately 72,000 years (Zeuner, 1970) which gives a minimum age for the valley. The Middle Pleistocene - Upper Pleistocene boundary at approximately 187,000 years places a maximum age. Late

Acheulian stone industries of inter-Kamasian - Gamblian times have been dated at 115,000 years (Zeuner, 1970).

Although Bishop (1971) mentions the age of 9000 years, this is in connection with the Naivasha basin in Kenya and not with the Albertine rift. One must therefore conclude that White (1985) has misinterpreted Bishop and that the maximum age for Bwambag is probably much older than 9000 years.

This last section on the geological history of the Semliki valley is being prepared for a joint publication with Mr. Kevin Walsh, Department of Geology, University of the Witwatersrand.

APPENDIX IV

THE USE OF CHROMOSOMES AND *ELECTROPHORESIS* FOR THE *IDENTIFICATION* OF MEMBERS OF THE ANOPHELES GAMBIAE COMPLEX.

The members of the Anopheles gambiae complex were defined originally by differences in the mating characteristics of the species (Davidson et al., 1967). To determine what the mating types were, was a tedious and time-consuming process. The discovery that the giant polytene chromosomes, found in the salivary glands of the fourth instar larvae and the ovarian nurse cells of the adult females of the different species, were marked by species-specific paracentric inversions (Coluzzi 1968, Coluzzi & Sabatini 1967, 1968, 1969), meant that laborious laboratory cross-mating identifications could be dispensed with. Initially however, the obtaining of readable chromosome preparations from fourth instar larvae was not easy. The problem was somewhat simplified by Green (1970) who presented a chromosome map of the X chromosomes from the ovarian nurse cells of the adult females.

Anopheles quadriannulatus was chosen as the arbitrary standard (Coluzzi et al., 1979). The breakpoints of

the fixed inversion differences in the other species were recorded on a standard chromosome map (see figs. 3 & 4). The chromosomes of unknown individuals were then compared to these maps. Identification of individual females by this method is the most accurate available. Extensive sampling in Africa from 1967 to the present time has shown that the species-specific chromosome rearrangements are consistently reliable.

A second means of identifying members of the gambiae group is the visualising of allozymes using horizontal gel electrophoresis. This method is rapid but its accuracy depends on a knowledge of the mobilities of diagnostic allozymes in the population under study. Mahon et al. (1976) published a detailed explanation of the use of enzyme electrophoresis for the identification of species in the gambiae group. They tested three enzyme systems and found two to be of some value. Superoxide dismutase (SOD) was used to identify merus. Esterases 1, 2 and 3 were used to identify arabiensis, gambiae and quadriannulatus. They found the method 95% reliable. Miles (1978) included melas and bwambae in an extensive electrophoretic study (22 enzymes were considered) on the group over much of Africa. He produced a biochemical key (Miles, 1979) using the following systems:

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1) superoxide dismutase (SOD) - slow (95%) merus, very fast (105) bwambae;

2) glutamate-oxaloacetate transaminase (GOT) - slow (95) quadriannulatus;

3) octanol dehydrogenase (ODH) - slow (90/95) arabiensis, fast (100/105) gambiae;

4) esterase 1 (EST-1) - very slow (70/75/80) melas.

The probability of error was 0.002 for East Africa and 0.07 for West Africa.

Comparison of the use of the above techniques for identification purposes reveals some disadvantages of both. Chromosomal identification needs cytogenetic expertise and many workers find it difficult to follow the banding patterns and recognise fixed inversions. The collection of field material is made difficult by the fact that half-gravid females are required for this method. This can limit the sample size considerably. On the other hand, although field sampling for electrophoresis is very simple, sophisticated laboratory equipment is necessary to process the sample. More important, while the use of electromorphs to identify members of the group is probably one of the most convenient methods available, it must be realised that such identifications cannot

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% Indicates electromorph mobility (Mahon et al., 1976).

be absolute. The limitations of this method have been carefully described by several authors:

"The gene frequencies we have encountered in Rhodesia may not necessarily be representative of those found elsewhere in Africa." (Mahon et al., 1976).

"Measurements of genetic distance or similarity based on electromorph frequency data should be treated with caution, and not as a systematist's panacea. .... These values can only be of use if they are derived from taxa whose individual biological species or subspecies status has already been established." (Miles & Paterson, 1979).

"It must be emphasized that the probabilities of identifying species A and B correctly are estimates." (Miles, 1979).

"One or other of a pair of electromorphs with which assortative mating is established in one area may be absent, or at a low frequency, in populations representing the same two fields for gene recombination in another." (Miles, 1981).

"Genetical studies and particularly polytene chromosome investigations are still essential for a reliable identification of the members of the gambiae complex...." (Cambournac et al., 1982).

In the light of this, it is important that the

identification of a species be confirmed cytogenetically or by crossing experiments.

The sample of gambiae s.l. obtained from the island of Grand Comoros is an excellent example of how identifications should be made (Hunt & Coetzee, 1986b). Twenty out of 64 females were chromosomally identified as gambiae s.s. Fifty-eight individuals were identified electrophoretically as gambiae, based on the presence of the fast ODH band - a result confirmed chromosomally in 19 cases and once by cross-mating. The possibility that arabiensis, quadriannulatus and merus may also occur on the island could not be ignored. For this reason, gene frequencies of any other members of the group occurring in the area also need to be worked out. This would have to be done before diagnostic electromorphs can be used as the sole means of identifying gambiae group mosquitoes in the Comoros archipelago.

Not only is specific identification important in understanding malaria transmission in any given area, but, futhermore, various chromosomal inversions within the taxa arabiensis and gambiae are correlated with different behavioural traits in the vector populations in West Africa (Coluzzi et al., 1979). Some of these traits have important consequences for the control of

vector populations or their potential as vectors. For this reason, it is incumbent on entomologists working on this group to record inversions that occur in the populations they are studying in case these are subsequently shown to be correlated with important biological characteristics.

In conclusion, once electromorph frequencies for the gambiae group species in a given area have been worked out, electrophoresis can be used with confidence to identify them. However, until this is done, electrophoretic data must be correlated with chromosomal identifications.

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