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A taxonomic and genetical study of the Anopheles coustani group of mosquitoes in Southern Africa.

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any other signals or any combination of these signals. An incidental effect of SMRS is to compartmentalize gene pools. A direct study of the SMRS in anophelines is technically very difficult and at present largely impracticable, however, genetic variation within single samples from nature may reveal discontinuities which result from the lack of gene flow between two or more sympatric gene pools resulting from different SMRS's.

With the advent of such genetical studies, many species complexes have been revealed within the genus Anopheles and the use of the term sibling or cryptic species by taxonomists has become increasingly popular.

1.2 Sibling species

Sibling species are particularly common in nocturnal organisms which use non-visual signals in their SMRS's eg. auditory, chemical or tactile signals. Consequently, they are not easily differentiated using a standard classical taxonomic approach.

It was in the early 1920's that the first indications appeared pointing to species complexes within the genus Anopheles (Roubaud, 1920, Grassi, 1921). It was noticed that in some parts of Europe there was a curious absence of malaria where the vector Anopheles (Anopheles) maculipennis Meigen was nevertheless abundant. Differences

from place to place were noted in the adult feeding habits and larval breeding sites and two populations in Holland were referred to as "shortwings" and "longwings" because of a statistical difference in size between the two populations (Swellengrebel & De Buck, 1938). It was discovered by cross-mating experiments, that various populations were inter-sterile (De Buck, Schoute & Swellengrebel, 1934). Then it was noticed that egg patterns could be used to identify these biologically different populations (Hackett & Missiroli, 1935). Bates (1949) summarizes all the information known at that time. Essentially, the maculipennis complex was first resolved using behavioural and morphological characteristics. Later genetical studies confirmed these observations (see White, 1977).

In South Africa, the subgenus Anopheles is represented by the coustani group of species, relatives of maculipennis, which are highly anthropophilic and could be possible secondary vectors of malaria or arboviruses.

The first species complex to be resolved in purely genetical terms was that which included the African malaria vector Anopheles (Cellia) gambiae Giles. It was suggested that gambiae was, from laboratory cross-mating experiments, actually a complex of species (Paterson, 1962, Davidson, 1962, Kuhlow, 1962). The following year, field studies along the Sabi river, Zimbabwe, at

Chirundu, Zambia and in Swaziland revealed another fresh-water member of the complex (Paterson et al, 1963). Later, by using fixed inversion differences in the banding sequences of the giant polytene chromosomes found in the fourth instar larvae of the gambiae complex, it became possible to identify the different members cytologically (Coluzzi & Sabatini, 1967, 1968, 1969). Unfortunately, to this day, there is still no reliable way of identifying the different members of the gambiae complex morphologically and all identifications are made either chromosomally or electrophoretically. The morphological studies so far carried out (eg. Coluzzi, 1964, Reid, 1973, 1975a, 1975b) have all been done on colony material subject to inbreeding and artificial selection pressures as well as artificial environmental influences. Their findings have not proved relevant to wild material.

1.3 Objectives

For practical purposes, morphological identification of malaria vectors is of the utmost importance. While genetical techniques are valuable in the laboratory, they are impracticable in the field where sophisticated equipment cannot conveniently be used. It is therefore important, wherever possible, morphological characters should be sought using genetically identified material from the field.

The coustani group was chosen for this study because

they are highly anthropophilic and are thus potential vectors of disease. The study of the group as a whole, using genetical theory and data from the chromosomes (modified technique of Kanda (1979) for fourth instar larvae), starch-gel electrophoresis (Mahon, Green & Hunt, 1976, Miles, 1978) and morphology (Belkin, 1962), was carried out to provide an intergrated picture of use to malaria epidemiologists.

Only wild-caught material has been used, thus eliminating the drawbacks of work with inbred laboratory colonies. At the same time, due to the difficulty of rearing and colonizing these mosquitoes in the laboratory, extensive cross-mating experiments were not possible, though some were performed.

It is hoped that this study will show the value of using the different approaches simultaneously and in a co-ordinated way in the study of a species complex.

1.4 A brief history of the Anopheles (Anopheles) coustani group of mosquitoes

Anopheles coustani was first described from Réunion by Laveran in 1900. Anopheles tenebrosus was described from Egypt by Dönitz, and Anopheles ziemanni from Cameroun by Grünberg, both in 1902. In later publications, the synonym of mauritianus de Grandpre & de Charmoy was used

for coustani and the above two latter species were regarded as "varieties" thereof (Edwards, 1928). When the name of "coustani" was reinstated, tenebrosus and ziemanni became "varieties" of it (De Meillon, 1947). In 1968, Gillies & De Meillon in their monograph on anophelines of the Ethiopian geographical region, elevated both varieties to specific rank on evidence from morphological differences and geographical distribution. All three are sympatric over a wide range of their distribution but show no intergrades in adult morphological characters. All three are both zoophilic and anthropophilic feeders and a small number have been shown to contain sporozoites of human Plasmodium species in the salivary glands (see Gillies & De Meillon, 1968). Their breeding habitats are very similar, largely in swampy, vegetated areas.

However, all authors record very few differences between the immature stages of these species (Evans, 1938, De Meillon, 1947, Gillies & De Meillon, 1968) except for egg characters where Gibbons (1933) describes the egg of ziemanni but mentions no polygonal markings on the ventral surface of the exochorion which is so typical of the other two species. The authors mentioned above did not refute this description. Evans (1938) also mentions a slight variation in one pupal character of ziemanni found in East Africa, and Vincke & Parent (in De Meillon, 1947) mention a pupal variant of tenebrosus.

It was perhaps the lack of differential characters in the larvae and pupae of the three species that prompted Gillett (1972) to postulate that the three might actually belong to a single polymorphic population even though the distribution of the forms was not in accordance with the expectations for a single polymorphic species (Ford, 1940).

CHAPTER TWO

THE CHROMOSOMAL ANALYSIS

2.1 Introduction

Polytene (Multistranded) chromosomes are the product of endomitosis, which consists of many cycles of chromosome duplication without nuclear division and with tight synapsis between homologous chromosomes. Appropriate staining techniques bring out the banding patterns along the length of the polytenes. The recognition of sibling species has often been aided by detecting differences shown by these banding patterns (Stegnii & Kabanova, 1978, Lambert, 1979, Green & Miles, 1980). Frizzi (1947) with his study of salivary gland polytenes from fourth stage larvae and Coluzzi (1968) with the polytenes of ovarian nurse cells of adult female anophelines, showed the importance of banding sequence differences for routine identification of species complexes. Paterson (1964) used polytene chromosomes in searching for natural hybrids in the Anopheles gambiae complex at Chirundu.

The use of polytene chromosome analysis in evolutionary studies has been reviewed by Dobzhansky (1970) and White (1973).

2.2 Methods

2.2.1 Mosquito collecting and rearing techniques

Female mosquitoes were mainly caught biting man or in man-baited nets, from various localities (see Appendix I). Individuals were identified on hind leg bandings (fig. 1), offered a blood meal and then put aside for egg-laying using the hanging-net method (Coetzee & Meiswinkel, 1979). Each egg batch thus obtained was treated as a unit or "family" and kept separate from other batches. The larvae were cultured collectively in distilled water and fed on finely ground dog biscuits. When they reached the fourth instar, a minimum of 10 per family were isolated in individual tubes for pupation and adult emergence. This method ensured that the larval and pupal pelts were correlated with the adult. The fundamental importance of this procedure will be discussed below (chapter 4).

A minimum of five fourth instar larvae were sacrificed for their salivary glands, and at least two adults stored in liquid nitrogen for subsequent electrophoresis.

Eggs were collected on filter-paper soon after the larvae had emerged, and air-dried for later examination.

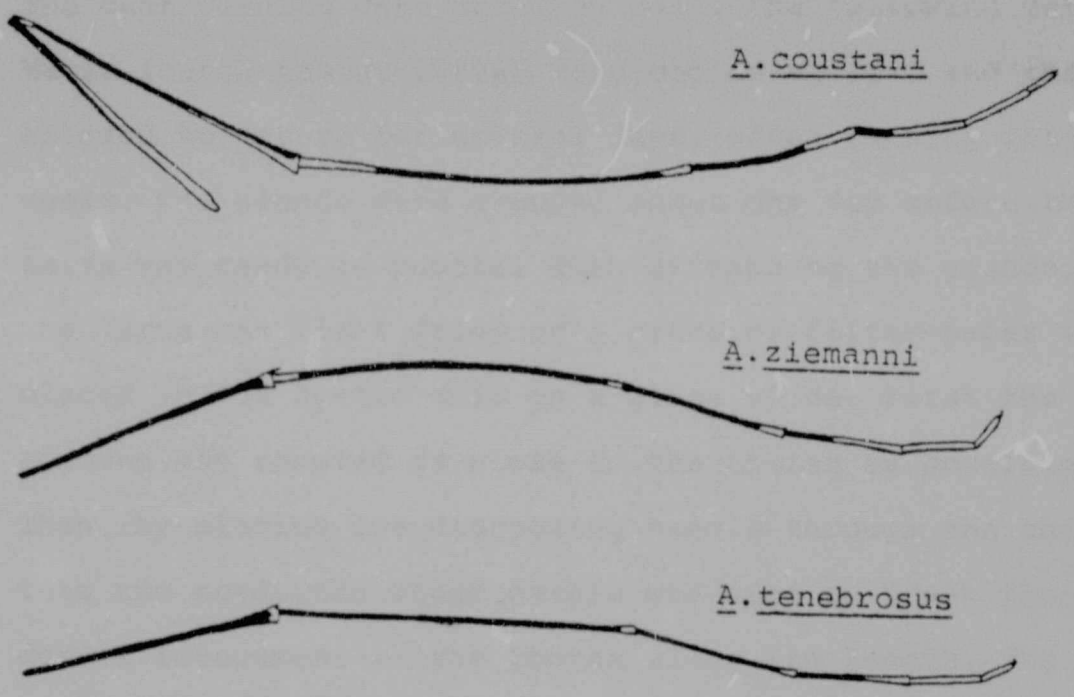


Fig. 1. The hind legs of the three species A.coustani, A.ziemanni and A.tenebrosus showing the differing amounts of pale areas

2.2.2 Salivary gland polytene chromosomes technique

Due to the absence of usable polytene chromosomes in the ovarian nurse cells of these mosquitoes, it was necessary to study those found in the salivary glands of the fourth instar larvae. French et al (1962) and Kanda (1979) both describe the technique for salivary gland preparations but as neither proved very successful, modifications

had to be made.

The best results were obtained using the following method. Early fourth instar larvae were cooled to 15°C and then allowed to mature for several days, often running into weeks. The glands were cropped about one day before the larva was ready to pupate. When extracting the glands, the larva was first dried on a piece of filter-paper then placed in 15% acetic acid on a glass slide. First the abdomen was removed as close to the thorax as possible. Then, by placing one dissecting needle through the thorax into the head, the other needle was used to break the dorsal integument of the thorax along its length. The head was then severed and the diverticulum and oesophagous removed. The opened thorax was placed in a drop of 1% sodium citrate to halt fixation while further dissections were carried out. This also seemed to make the salivary glands more conspicuous. The dissected glands were placed in Carnoys fixing fluid (1 part Glacial acetic acid, 3 parts absolute ethanol, freshly mixed) at least overnight.

The fixed glands were placed on a siliconized coverslip and most of the Carnoys removed. A tiny drop of lacto-acetic orcein (1g orcein in 25ml glacial acetic acid and 25ml 85% lactic acid, diluted 1 part in 3 parts 50% propionic acid) stain was applied for $\pm$ 1 minute. Two to three drops of 50% propionic acid was added and a slide dropped onto the coverslip which adhered by capillary

action. The coverslip was judiciously tapped to spread the chromosomes. The final preparation was heated to obtain perfectly flat spreads suitable for photomicrography. Slides were stored in a fridge in dishes thinly awash with 50% propionic acid and were useful for several months (C.A. Green, pers. comm., has said that he has kept slides in this manner for over one year and still found them usable).

Photography was carried out on a Vickers phase-contrast microscope (X1000 magnification) using Kodak Panatomic X film and exposure adjusted to three seconds. The film was developed in Kodak D19B for five minutes. The negatives were printed on Ilfospeed grade 2 paper and developed in Kodak D193 for two minutes.

Preparations of polytene chromosomes from salivary glands of anopheline larvae do not approach the quality of those obtained from ovarian nurse cells in the adults. There is also a difference in quality between the subgenera with Anopheles salivary gland polytenes being much poorer than those of Cellia (Baker & Kitzmiller, 1965, Coluzzi et al, 1970, Seetharam & Chowdaiah, 1971). However, preparations were obtained which enabled montages to be made for chromosome maps.

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2.3 Cytological characteristics

2.3.1 Chromosomal species

A total number of 114 families of the various species were analysed and fixed chromosomal inversion differences indicated that coustani and ziemanni each comprised two species at least. A.tenebrosus revealed no intra-specific chromosomal differences but the sample size was small.

Five or more individuals per family were used for this study because it took, on the average, this number to obtain a single scorable preparation. In one case, a whole family was used without a single usable chromosome spread being obtained.

The nomenclature used here for arm designations is the one generally in use, which shows arm associations within species and does not reflect homology of arms between species or groups of species (eg. Coluzzi & Sabatini, 1967). The shortest arm is the X and the autosomal arms are designated as 2R, 2L, 3R and 3L. The newer method of Green & Hunt (1980) was not used as it still only applies to ovarian polytenes and associations have not yet been worked out to correspond with the salivary gland polytenes or to correlate subgenera.

The chromosome maps were made according to the method of Stalker (1964) and used to check banding sequences of unknown specimens through the microscope according to Carson's method (1970). The maps of coustani (fig. 2) and ziemanni (fig. 3) show inversion differences between the sibling species, here designated as coustani A and B, and ziemanni A and B, as well as floating inversions occurring within each species. A.coustani A and ziemanni A were chosen as arbitary standards respectively. Fig. 4 is the complete map of tenebrosus.

Easy identification of the four species coustani A and B, tenebrosus and ziemanni s.l. can be made from the fixed inversion differences on the X chromosome as shown in fig. 5, but to differentiate between ziemanni A and B, one must use the inversions on arms 2L and 3R (fig. 3).

Inversion notations follow that of Coluzzi et al (1970). For example, the section of chromosome between the markers "a" on arm 3R of the standard ziemanni A arrangement (fig. 3) is fixed for that species and is denoted as $3Ra^{+}$. If this section was reversed it would be written as 3Ra. The heterozygote inversion is shown as $3Ra/a^{+}$.

2.3.2 Floating inversion polymorphisms

No floating inversions were seen in the samples of

Fig. 2. The chromosomal map of A.coustani A showing the fixed inversion difference with species B on the X arm and various floating inversions of both species on the autosomal arms.


3L
3R
2L
2R
X
c
c
a
b
b
a
a
a
a

Fig. 3. The chromosomal map of A.ziemanni A showing the inversion differences found in species B.

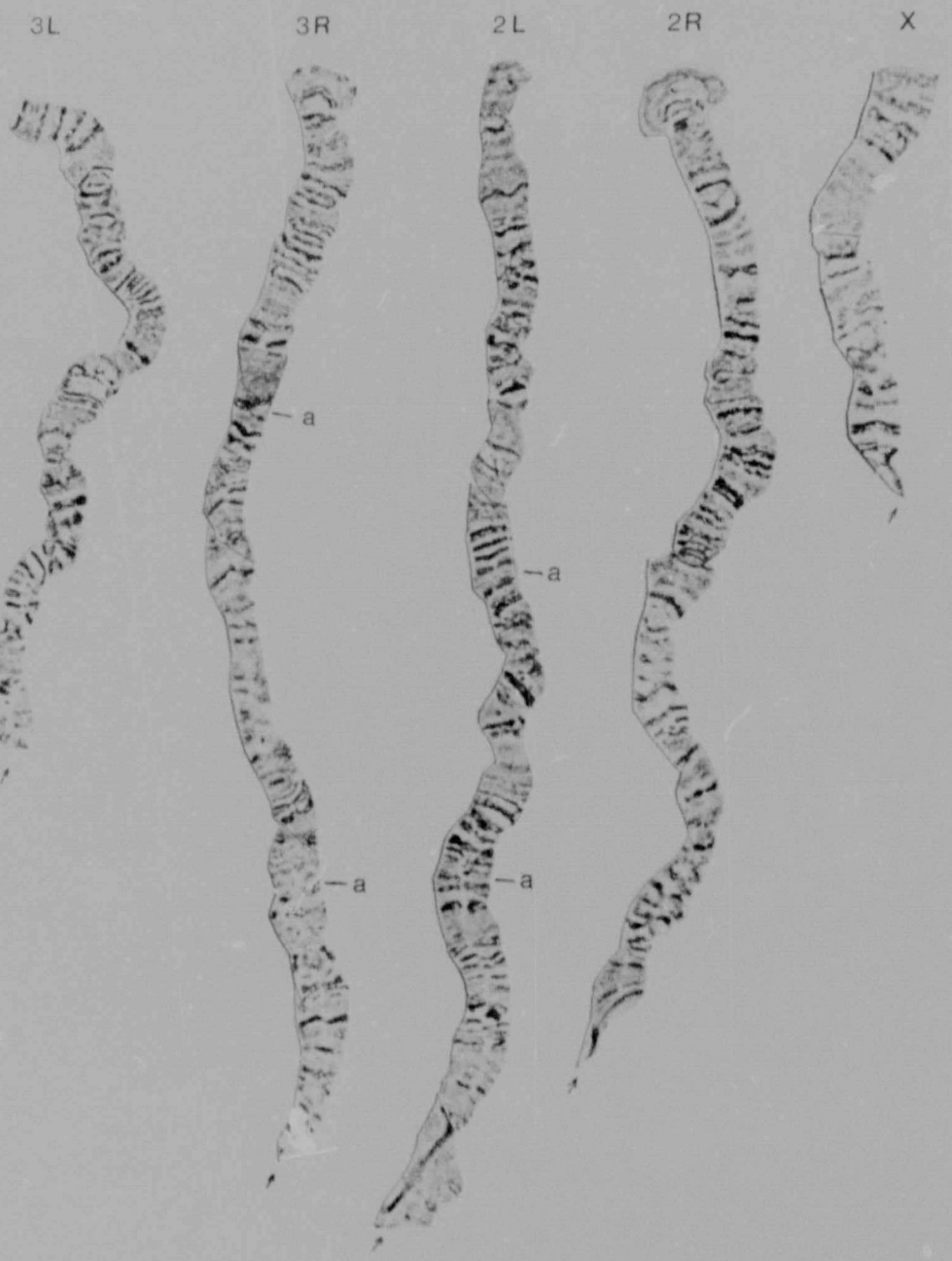
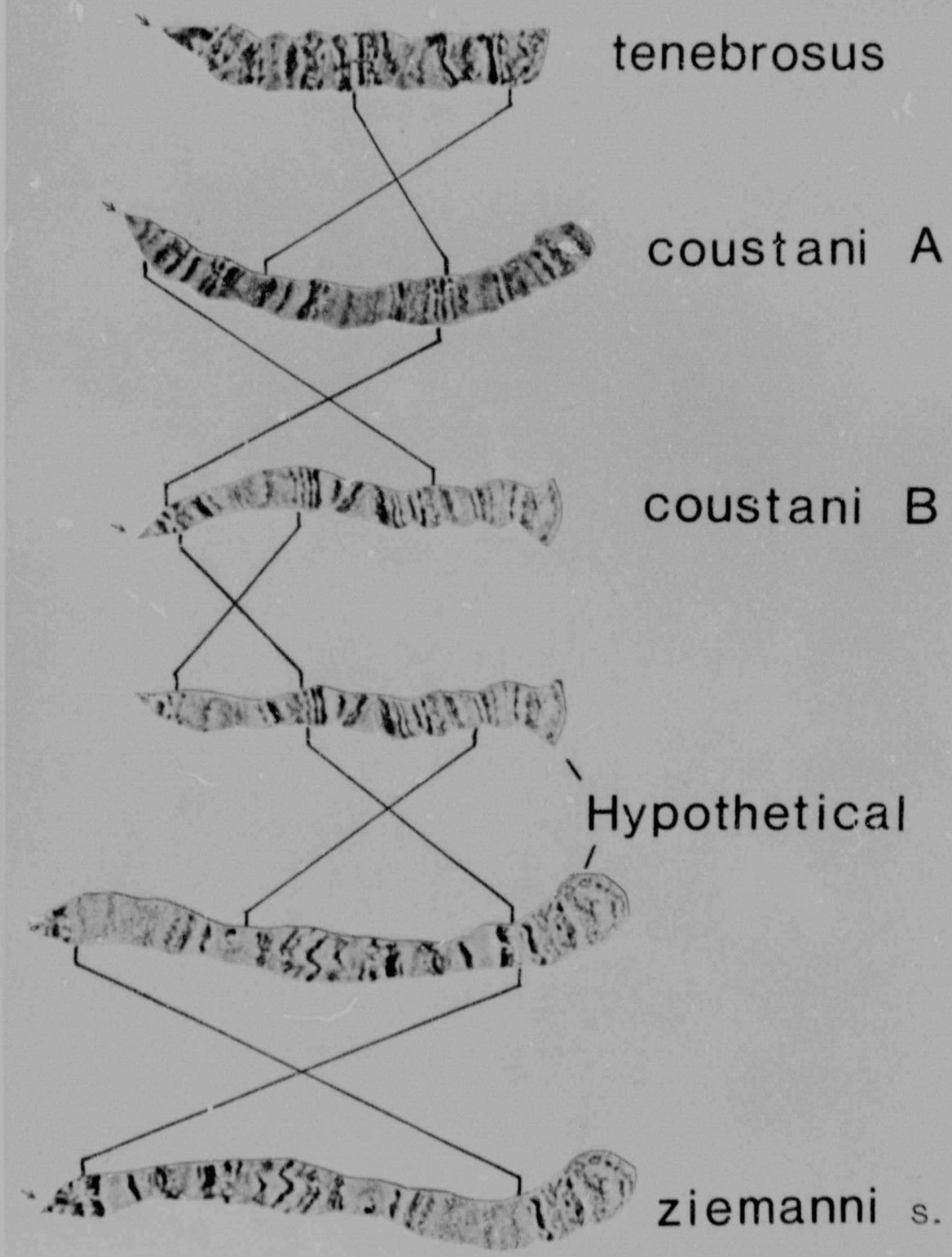

3L
3R
2L
2R
X
a
a
a

Fig. 4. The chromosomal map of A.tenebrosus.


3L
3R
2L
2R
X

Fig. 5. Fixed inversion differences on the X chromosome of four species belonging to the A.coustani group.


tenebrosus
coustani A
coustani B
Hypothetical
ziemanni s. l.

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