

Cytogenetic Evidence of a Possible Fourth Cryptic Species

Within the Taxon *Anopheles marshallii* (Theobald)

(Diptera: Culicidae) from Northern Natal

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ABSTRACT. Previous cytogenetic analysis of populations of *Anopheles marshallii* from localities in Zimbabwe and South Africa has revealed the existence of three geographically widespread species within the taxon. A further study, reported here, of individual females from Kosi Bay in Northern Natal has now uncovered another chromosomally distinct population. It is probable that this population represents a fourth species which is closely related to species B and C of the complex. The sex chromosome banding pattern of this new population is presented and its phylogenetic relationship to other species of the complex is considered.

The *Anopheles marshallii* complex is a group of species distributed over large areas of Africa. A previous study (Lambert 1979) has shown that the complex comprises at least three species which are widely distributed over Zimbabwe and South Africa. These three species are chromosomally distinct, in that the polytene chromosomes from ovarian nurse cells show a large number of fixed inversion differences in both the autosomes and the sex chromosomes. Morphologically, however, individual females of these species satisfy the description *Anopheles marshallii* and are extremely difficult to separate at the adult stage. They can most appropriately be described as cryptic species. Some evidence that the species differ in their biting behavior has been presented (Lambert 1979).

This report presents the results of further collections made from Zululand, South Africa. A number of *Anopheles* species found in the lowland coastal regions of South Africa are not found in other areas and it was considered important to collect individuals of *Anopheles marshallii* from this region.

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MATERIALS AND METHODS

Female mosquitoes were collected for ovarian polytene chromosome analysis from Kosi Bay, Northern Natal, South Africa (26°57'S; 32°49'E) on 21-5-79 (see Fig. 1). Those which were not already blood fed were given blood meals. After the female had taken a full blood meal she was left for approximately 30 hours until "half-gravid." This length of time is however quite variable depending on the ambient temperature and such factors as the availability of water for the mosquito. When the female was at the half-gravid stage, as judged by the development of the ovary and the digestion of the blood meal through the body wall, the ovaries were dissected out using a pair of fine forceps. These were then placed in a small vial of "carneys" fixative (1 part glacial acetic acid to 3 parts absolute alcohol) for at least 24 hours. Ovaries were always kept either in a refrigerator or wrapped in wet cotton wool. This whole process was carried out in the field. Bodies of some adult females from Kosi Bay are held in the Department of Medical Entomology, South African Institute for Medical Research.

In the laboratory ovaries were placed on a clean microscope slide in a 50% aqueous solution of propionic acid until the ovary swelled to approximately twice its original size, this process taking approximately one minute. Extraneous material was then dissected away and the 50% propionic acid was removed using filter paper. A drop of diluted acetoorcein stain was then added to the preparation. The stain of French et al. (1962) was diluted one part in three with 50% propionic acid. The ovary was then macerated using dissecting needles and left for a few minutes for proper penetration of the stain. This was then blotted up with filterpaper and washed twice in 50% propionic acid. A large amount of 50% propionic acid was then placed on the preparation and then a siliconized coverslip placed over the top. This was then tapped with a blunt needle and the preparation placed on a hot plate set at approximately 30° C. This aids in the spreading out of the arms of the polytene chromosomes. The ovarian polytene technique was originally developed by Coluzzi (1968) and further modified and developed by Green (1972), Hunt (1973), and Green & Hunt (1981).

Slides were stored in dishes containing a thin layer of 50% propionic acid and kept in the refrigerator at approximately 5° C. Preparations stored in this manner are useful for up to one month. Photography was carried out using phase contrast illumination and a green filter with a Leitz SM-LUX microscope. Panatomic X film with an ASA rating of 32 was used at an ASA reading of 100 and developing time in Kodak D-19 developer was increased from 3 to 4 minutes. This process enhanced the contrast of the final prints. The polytene chromosomes of species of the *Anopheles marshalli* group proved to be of a useful quality although not of the same quality as many other *Anopheles* species, e.g., those of the *Anopheles gambiae* complex.

RESULTS AND DISCUSSION

All 35 individuals collected from Kosi Bay were homosequential for the sex chromosome banding patterns. This pattern is shown (E) in Fig. 2 in comparison with the sex chromosome patterns of species B and C. The banding sequences are

represented by letters of the alphabet. Species B is used as a standard and the other banding sequences illustrated are compared to this standard species B sequence. The three banding sequences differ by a number of fixed sequence changes. No floating inversions have been found on the sex chromosomes of species of the *Anopheles marshallii* complex. Indeed it was previously reported that only species A showed such floating inversions on the autosomes (1980). Two inversions on chromosome 5 (designated 5a and 5b) were found in this species (Lambert 1980). Since during the course of a cytological study covering the period 1976-1979, 1393 wild-caught females were chromosomally analyzed, and it appears that, if floating inversions are present on the sex chromosomes they must be exceedingly rare.

The species B banding pattern shown in Fig. 2 differs from the banding pattern of females collected from Kosi Bay by one inversion - that involving regions A and B. Kosi Bay females are homosequential with species B females in the regions C - H. No species B females have been found to possess a floating AB inversion. Such an individual would be obvious because of the possession of a typical heterozygous loop on the X chromosome. Owing to the poor quality of the preparations no autosomal relationships were able to be estimated.

All this suggests that the females from Kosi Bay represent a fourth species of the *Anopheles marshallii* complex. The species, temporarily designated species E, appears to be fixed for a paracentric inversion in the A-B region. It should be pointed out that the evidence presented previously regarding the status of species A, B and C was unequivocal. This is because of a complete lack of hybrid individuals in areas where two or more of the species are sympatric. The population being considered here appears to be allopatric and hence this absolute test for the lack of hybrids cannot be made. Therefore, until such time as an area of overlap with other species is found a certain amount of caution is needed. However, it is felt that, in view of the complete lack of floating inversions found on the X chromosome of species of the *Anopheles marshallii* complex over very large areas, it is unlikely that this population represents purely a geographic isolate which has one arrangement fixed. As supporting evidence for this conclusion Lambert (1979) has found enzymatic and morphometric differences between the species A, B and C and the population from Northern Natal.

It should be noted that species B and C must have been connected by a hypothetical population since two overlapping inversions are necessary in order to derive the species C pattern from species B. This hypothetical intermediate sequence is represented in Fig. 3. The banding pattern of species E individual is the same as this hypothetical sequence. Fig. 4 represents two possible relationships between the species based on these data. Species E may have been derived from a hypothetical ancestor and the speciation event occurred without any change in the chromosomal banding sequence. Alternatively species E may be a direct intermediate between species B and C.

The taxonomic significance of these findings is not immediately clear. Currently three synonyms of *Anopheles marshallii* are recognized. These are *Anopheles transvaalensis* (Carter), *Anopheles pseudocostalis* (Theobald) and

Anopheles pitchfordi (Giles). *An. pitchfordi* was described from the type locality 80 miles north of Eshowe, Northern Natal. This form was described by Giles (1904) as being paler than the type form. The present taxonomic position of *pitchfordi* is confused. Evans (1927) reduced it to a variety and Vincke et al. (1957), later showed a complete range of variations between pure *pitchfordi* and *marshallii*.

Since collections reported here of species E are also from Northern Natal the question of whether species E is *An. pitchfordi* is an obvious one. Preliminary evidence from discriminant function analysis involving the type specimen of *Anopheles pitchfordi* indicates that this specimen does not belong to either species A, B or C of the complex, nor does it apparently belong to species E. A larger sample is obviously needed. However, the possibility that even more species exist within the taxon *Anopheles marshallii* must be a serious possibility. The area of Northern Natal is an important locality for future studies on this interesting species complex.

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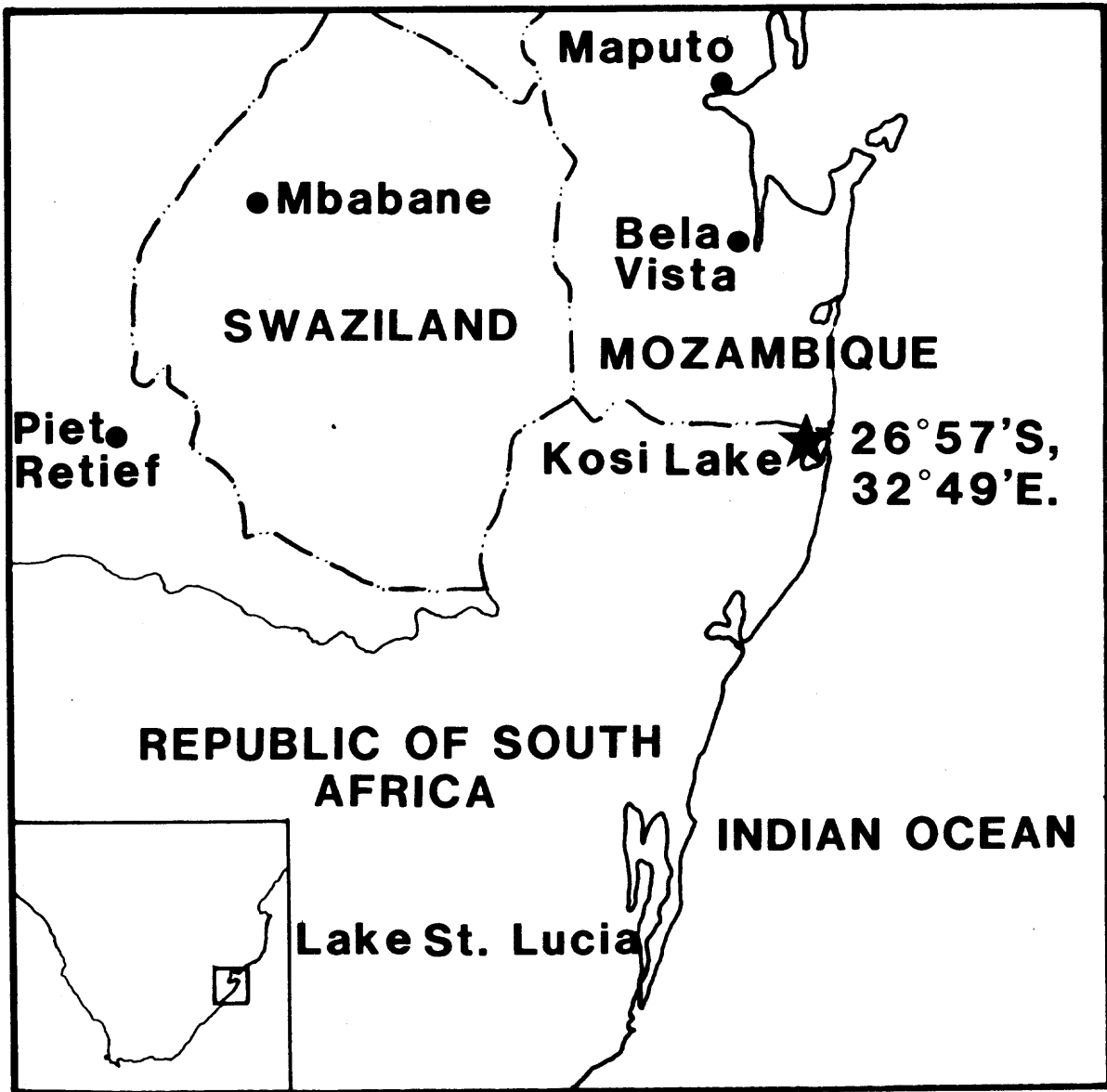


Fig. 1. Collection site of 35 females of *Anopheles marshallii* used in cytological analysis

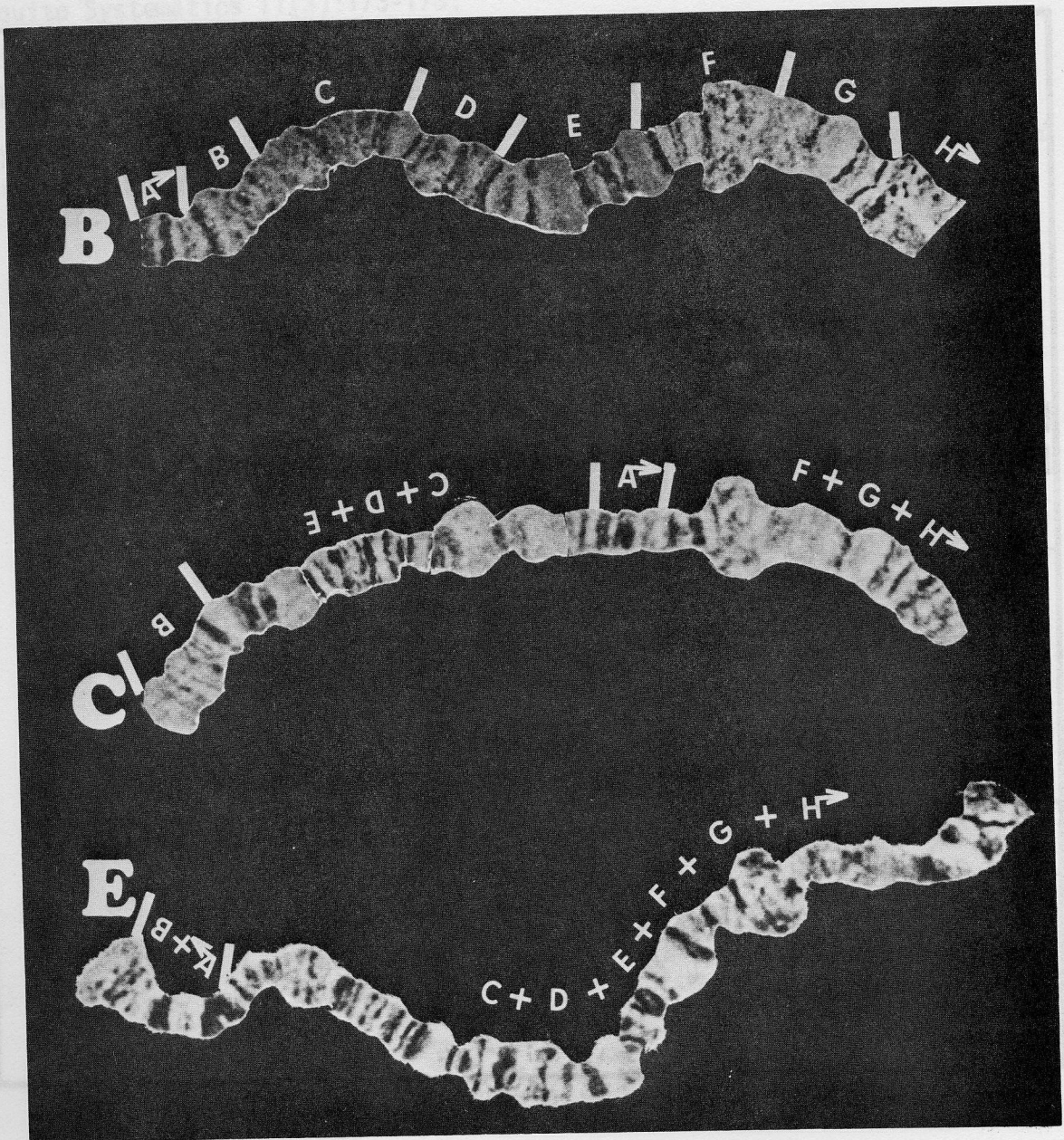


Fig. 2. Composite photographic maps of the sex chromosomes of species B and C of the *Anopheles marshallii* complex, together with the banding pattern of individuals from Kosi Bay (labelled E)

