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INTERPRETATION OF VARIATION IN OVARIAN POLYTENE CHROMOSOMES OF ANOPHELES FUNESTUS GILES, A. PARENSIS GILLIES, AND A. ARUNI?

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A photomap of polytene chromosomes from ovarian nurse cells of *Anopheles funestus* Giles is presented. A new system of arm designation is introduced for the subgenus *Cellia*, which unlike the current system, permits a unified inversion nomenclature between species' groups. The arrangement of *A. funestus* chromosomes is arbitrarily chosen as standard, and *A. parensis* Gillies and *A. aruni?* (de Meillon *et al. Mosquito News* 37: 657-661, 1977) are compared to it in terms of fixed inversions and floating inversions in present-day populations. *A. parensis* differs from standard by two fixed inversions, and *A. aruni?* is homosequential with the *funestus* arrangement. The relationship of fixed inversion differences and speciation events is discussed in the light of homosequential species. The distribution of floating inversions on arm 2 in *funestus* and *aruni*? offers a possible example of Wallace's (1959) 'triad' hypothesis (*Cold Spring Harb. Symp. quant. Biol.* 24: 193-204).

Introduction

Anopheles funestus is an important vector of human plasmodia in Africa, hence it and its close relatives have been subjected to intense taxonomic study. The three species reported here differ from each other morphologically only in the adult stage of the life cycle. In *A. parensis* the diagnostic characters in females occur only in 70 – 75% of individuals (Gillies & de Meillon, 1968 p. 148). De Meillon *et al.* (1977) reported the discovery of the South African species and informally referred to it as *A. aruni*?. Within their data sets for *A. funestus* and *A. aruni*? they show no overlap for the diagnostic characters they report, but the data set for *A. funestus* is small and there is a need for a more extensive study before their conclusions can be regarded as secure. It is worth mentioning that these differences, in both cases, are slight and formerly would never have been considered sufficient to recognise the specific distinctness of these taxa. Both species were recognised initially from behavioural data after the removal of the domestic, vector species by means of spraying houses with insecticide. The residual '*funestus*'-like populations tended to remain outside houses, and did not bite man in the case of *A. parensis* (Gillies 1962).

Recent experience suggests that little or no morphological divergence in anophelines has accompanied speciation events. This makes for insecurity in the recognition of specific taxa solely from morphological markers. A classical case in point is the *A. gambiae* species' group which includes other potent vectors of malarial parasites in Africa. These species are currently recognised from the fixed inversion differences between them (Coluzzi & Sabatini, 1967, 1968, and 1969). Morphological divergence is very slight, and in the case of *A. gambiae, A. arabiensis*, and *A. quadriannulatus* (informally designated in literature cited here as species A, B, and C respectively) none has yet been reported (Coluzzi 1964).

This paper reports on the cytogenetics of *A. funestus* and its closest relatives.

Material and methods

A. funestus adult females were collected from the following localities. Kanyemba (15°40'S: 30°20'E, N = 556) and Binga (17°45'S: 27°25'E, N = 68), Zimbabwe/ Rhodesia; the Okavango River (18°03'S: 21°39'E, N = 153), Namibia; the Jos Plateau (N =

26), Nigeria; Msihu Ragwe 72 km south of Mombasa (laboratory reared F_2 of a single family), Kenya. *A. parensis* was collected from Salisbury (17°48'S: 31°04'E, N = 11), Zimbabwe/Rhodesia; Makanes Drift (27°02'S: 32°19'E, N = 67), Sihangwane (27°05'S: 32°30'E, N = 20), Zululand/South Africa. *A. aruni*? came from around Tzaneen (23°48'S: 30°10'E, N = 54), Dzindi (22°59'S: 30°26'E, N = 10), and Komatipoort (25°26'S: 31°56'E, N = 9), South Africa.

Blood-fed females were offered a second meal early in the morning following their capture. They were then held at about 20°C for approximately 36 hours at a relative humidity in excess of 80%. Half gravid females were killed and ovaries cropped, and stored individually in Carnoy's fluid at low temperature.

On return to the laboratory chromosome spreads were prepared according to the method in Green (1972), and Hunt (1973) but with the important addition of heating squash preparations at about 60°C for 1 to 2 minutes. This latter procedure greatly increases squashing and reduces the depth of field such that all parts of a field are usually in focus at about x 1000 under the microscope. Photographs were taken on 35 mm Gevaert Scientia 50B65AH film. Illumination of the film was about 8 lux for 6 sec. using phase contrast illumination with a final magnification on the film of x 500. Film was developed in Kodak D19b developer for 5 minutes at 20°C, and printed on normal paper to a final magnification of x 4000. This size is convenient for handling prints despite the 'empty' magnification beyond about x 2000.

Large collections of prints of each chromosome arm were made showing them in various degrees of stretching. The photomap (Fig. 1) was made according to the method of Stalker (1964) and, as he points out, serves primarily as a book keeping device to locate inversion breakpoints. Interspecific homologies are rarely detectable from single, photographic editions of chromosomal elements. The map was used to check banding sequences of unknown specimens through the microscope according to Carson's method (1970).

Crosses between families from wild-caught *A. aruni*? from Tzaneen (N = 3) and *A. funestus* caught biting man inside houses at Kanyemba (N = 4) were made by forced mating (Baker *et al.*, 1962). F_1 males were dissected in saline and their internal genitalia observed using phase contrast optics. Ovarian polytene chromosome preparations were made from F_1 females.

Results

Proposed new nomenclature for arm designation

Coluzzi *et al.* (1970) in describing and relating the polytene chromosome rearrangements in *A. superpictus* and *A. stephensi*, point out that the four autosomal arms differ in their associations from that found in the *A. gambiae* species group. They suggest that a translocation event may be responsible for this difference. The species reported here show yet a third arm association (see Fig. 1), presumably due to a second translocation event (the *funestus* group belong to the series Myzomyia). Furthermore close relatives of *A. funestus* which we have studied, *A. leesoni*, *A. rivulorum*, and *A. fuscivenosus* show the arm association reported by Coluzzi *et al.* (1970) for the series Neocellia, which is shared by species in the series Myzomyia reported in the literature (Chowdaiah & Seetharam 1975, Saifuddin *et al.* 1978). Using the current means of arm designation we cannot produce an efficient, unified inversion-notation within the *funestus* group as a whole. The reason is that the current nomenclature is designed to show arm associations within species, and does not reflect homology of arms between species. Thus workers have recognised 2R throughout all species studied, and so designated this arm, which obliges one to designate other homologous arms in different species with different terms. Table 1 shows the current situation, and what would happen if we were to use the current system to designate the chromosome arms of *funestus*. We have used the arm designation of *A. gambiae* as an arbitrary standard. Thus each species group is shown in terms of homologous elements with the *gambiae* designation in Table 1. Hyphens show arm associations, and italics show the designations actually given by authors as dictated by the current system. We also report arm associations of species previously unpublished in Table 1.

Thus we introduce a new system in this paper which gives each arm a unique designation independently of its particular association in the group under study. Chromosome arms of *A. funestus* homologous with *A. gambiae* are as follows: *gambiae* 2R = 2, 2L =

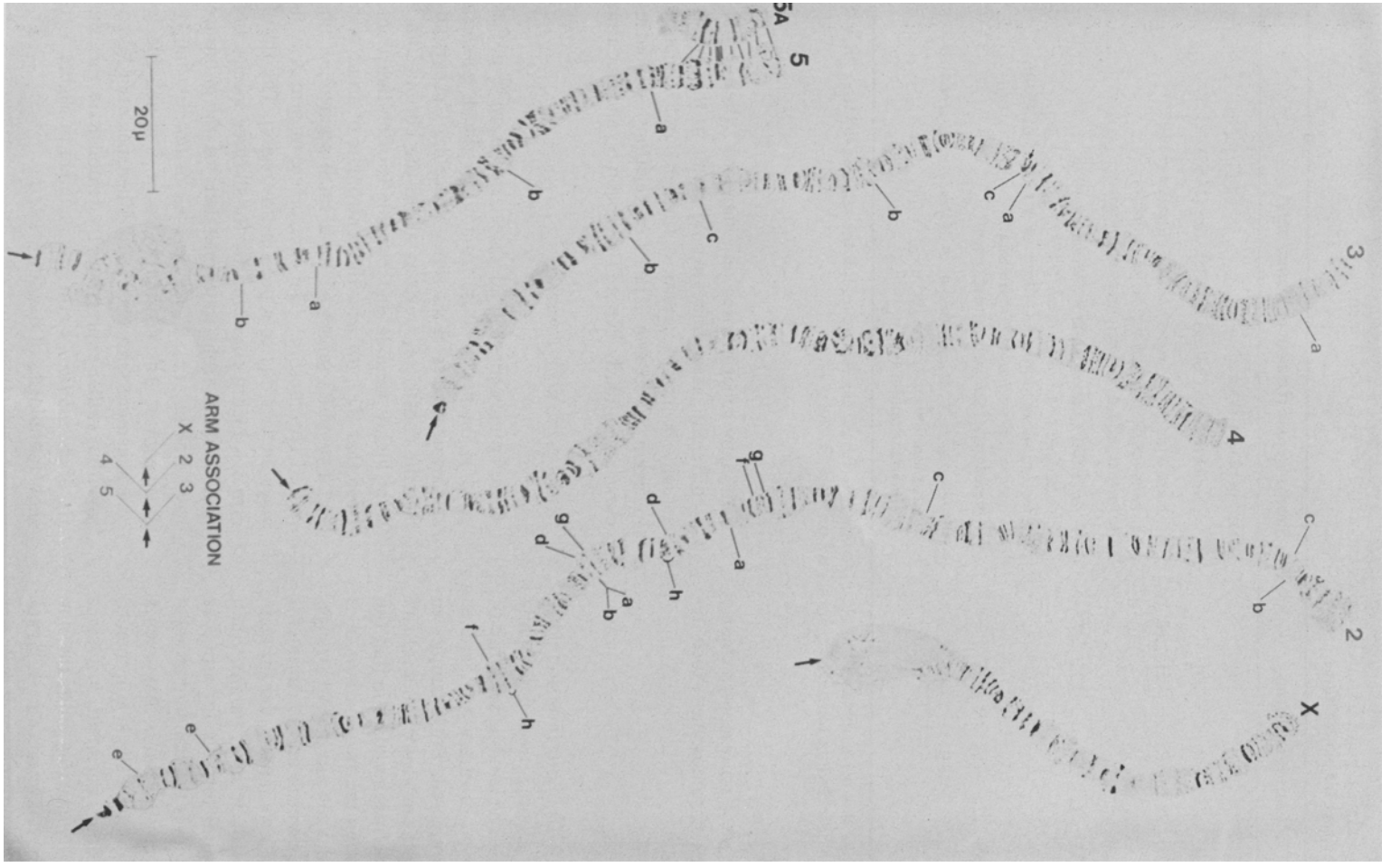

Fig. 1. The ovarian polytene chromosomes of *Anopheles funestus* Giles. Specific inversion formulae are *A. funestus*, X, 2 a/+ b/+ c/+ (d/+?) e/+ h/+, 4, 3 a/+ b/+, 5 a/+ b/+; *A. aruni?*, X, 2 ad/++, 4, 3, 5; *A. parensis*, X, 2 cg f/+, 4, 3c, 5. Arrows indicate centromeres. 5A is the tip of arm 5 from *A. culicifacies* and the lines indicate homologous bands between it and *A. funestus*.

Table 1

Homologous autosomal arms of ovarian polytene chromosomes within the subgenus *Cellia* in terms of the designation used for the *A. gambiae* group of species (see text for explanation).

Series of species	Autosomal arms								Species studied (Ovarian polytenes only)
	2		3		4		5		Proposed new system[+]
Pyretophorus	2R	–	2L		3R	–	3L		*A. gambiae* group Coluzzi and Sabatini (1967)
Neocellia	2R	–	3L	*2L*	3R	–	2L	*3L*	*A. stephensi*, and *A. superpictus* Coluzzi *et al* (1970) *A. maculatus*, *A. annularis*, *A. maculipalpis* *A. rufipes*, and *A. pretoriensis* unpublished
Myzomyia	2R	–	3L	*2L*	3R	–	2L	*3L*	*A. fluviatilis* Chowdaiah and Seetharam 1975 *A. culicifacies* Saifuddin *et al* 1978 *A. leesoni*, *A. rivulorum*, *A. fuscivenosus*, *A. demeilloni*, *A. theileri*, *A. wellcomei* unpublished.
	2R	–	3R	*2L*	2L	*3R*	–	3L	*A. funestus*, *A. parensis*, and *A. aruni*?
Cellia and Paramyzomyia	2R	–	2L		3R	–	3L		*A. pharoensis*, *A. squamosus*, *A. cydippis*, and *A. argenteolobatus* unpublished. *A. cinereus* unpublished.

[+] Proposed new system used in this paper replaces the old nomenclature for *A. gambiae* chromosome arms as follows: 2R = 2, 2L = 3, 3R = 4, and 3L = 5 (arm associations for *funestus* are shown on plate 1).

3, 3R = 4, and 3L = 5. There is no reason to change the designation of the sex chromosome which remains arm X.

Inversion notation

We follow Coluzzi *et al.* (1970) in our inversion notation which they in turn adopted from Drosophila workers (e.g. Carson 1971). The banding sequence of one species is chosen as an arbitrary standard to which other species are compared. For example we have chosen *A. funestus* as the arbitrary standard and for arm 3 *A. parensis* differs from standard in that the chromosome segment between the two markers 'c' is reversed. We have not found the alternative arrangement, i.e. 'c' standard in *A. parensis* and so consider it fixed in that species. Should we have found the standard alternative together with 3c in *A. parensis* then we would write the structural formula for arm 3 as follows, 3c/+, where '+' denotes the standard arrangement. Another important convention is that when a sequence of inversions occurs on a single arm then to derive the particular specific arrangement, inversions should be made sequentially starting from the one nearest the arm designation. For instance in *A. parensis* 2f occurred on a 2g chromosome, and if 2f was artificially superimposed on a standard 2 then a totally different arrangement would result to that which actually occurs in nature. A further point is that an inversion 'a' on arm 2 bears no relationship whatsoever to a similarly denoted inversion on another arm.

Interspecific differences

The chromosomes of all three species were not as good in quality as those from either the *A. gambiae* species or species of the series Neocellia. The expanded and constricted regions along the chromosomes were highly variable and banding is often indistinct. Note that the puff near the centromere of arm 5 is in fact two puffs separated by a small segment of unpuffed chromosome. The centromere of arm X is always unclear due to a puff in this region. The tip of arm 5 is highly characteristic for all three species and quite distinct from other members of the group we have studied, i.e. *A. leesoni*, *A. rivulorum*, and *A. fuscivenosus*. The distinctive tip of 5 is not due to rearrangement with respect to other species of Myzomyia but rather expression. To demonstrate this, the tip of *A. culcifacies* arm 5 is included in Figure 1 to show homologies of banding pattern.

The structural formula for *A. parensis* is X,

2cgf/+,4,3c,5, and for *A. aruni*?, X, 2 ad/++,4,3,5. Arms are ordered to indicate their associations, i.e. arms 2 and 4 form one chromosome, and 3 and 5 the other autosome. The structural formula of *A. funestus* simply shows the floating inversions we have found in that species, X, 2a/+b/+c/+(d/+?)e/+h/+, 4,3a/+b/+,5a/+b/+. Several inversions are either rare in nature, or we had small numbers of insects. In these cases breakpoints shown in Figure 1 must be treated as approximations. Rare inversions are seen only as heterozygotes and it is technically difficult to determine breakpoints accurately in such individuals. They are as follows: 2b (no. of chromosomes, n = 8), 2c (n = 2) in *A. funestus*, (thus it may not be identical with the fixed 2c in *parensis*), and 5b (n = 2) from Kanyemba; 2e (n = 3) from Namibia; 2f (n = 2) from Salisbury. 2h was fixed in our Kenyan *funestus* material but our poor preparations did not permit more accurate determination than shown in Figure 1. (2h does float in Kenya, Miles pers. comm.). The uncertainty of 2d/+ occurring in *A. funestus* results from the complex nature of rearrangements in this segment. We first identified 2d/+ from Kanyemba, and all samples from that locality pre-dated the discovery and analysis of *A. aruni*?. Our initial scoring of the 2ad complex gave genotypic frequencies which departed significantly from those expected from the Hardy-Weinberg equilibrium. We could not score the several complex heterozygotes expected for the complex. We decided that the departure from Hardy-Weinberg equilibrium was most likely due to these technical problems and in further samples did not score this polymorphism. This was an unfortunate decision in the light of the subsequent discovery of *A. aruni*?.

Common polymorphic inversions

Table 2 gives the frequencies of common inversions floating in *A. funestus*. Only the Namibian sample deviated significantly from expected genotypic frequencies derived from Hardy-Weinberg equilibrium for 3a/+ ($\chi^2_{(1)} = 5.3$, $P < 0.05$). In that sample there was a relative excess of heterozygotes. Tests for homogeneity of genotypic frequencies between localities showed no significant difference between Kanyemba and Binga for $3^a/+^b/+$ ($\chi^2_{(2)} = 0.9$, $P < 0.7$).

Kanyemba and Binga were pooled and showed a significant difference when tested against the Namibian sample ($\chi^2_{(2)} = 70.0$, $P < 0.001$). The Nigerian sample is clearly different from the southern African samples, whilst the Kenyan sample could well have come from the southern African populations except for the presence of 2h. A detailed analysis of these common inversions and their relationship to indoor *versus* outdoor biting *A. funestus* will be presented elsewhere.

Table 2

Relative frequencies of the standard alternative for common polymorphic inversions in *Anopheles funestus* Giles

Locality	Sample size	3+a	3+b	5+a	2+a
Kanyemba	556	0.3964	0.4418	0.4851	
Binga	68	0.4265	0.3750	0.2868	
Namibia	153	0.6634	0.5817	0.4118	0.3856
Nigeria	26	1.0000	1.0000	0.7115	
Kenya	(5)	+	+	+	

(Kenyan sample F_1 from a single family, '+' indicates polymorphic)

The inversion 2ad/++ is commonly polymorphic in samples of *A. aruni*? and we could not score the complex heterozygotes. However in good spreads all heterozygotes appeared as complex. Since only two chromosome types occurred in homozygotes, i.e. 2ad and $2+^a+^d$ we assumed all heterozygotes were between these two types. Relative frequencies for chromosome 2ad were Tzaneen 1977, 0.71 (n = 21), Tzaneen, 1979, 0.29 (n = 33), Dzindi, 0.45 (n = 10), and Komatipoort, 0.61 (n = 9), where n = number of individuals scored. The 1977 sample from Tzaneen came from a single site and was collected over a period of one week, whereas the 1979 sample was accumulated over a two month period from several sites.

The gross morphology of internal genitalia from F_1 males in crosses between *funestus* from Kanyemba and *aruni*? from Tzaneen, appeared normal, unlike the results reported by de Meillon *et al.* (1977). However when Kanyemba females were crossed to Tzaneen males the hybrid males failed to show mature sperm in the vas deferens. Hybrid males of the reciprocal cross showed mixtures of mature and immature sperm. Figure 2 shows a montage of ovarian polytenes from an F_1 female. The high level of asynapsis was typical of 32 hybrid females examined, and is similar to that seen in interspecific crosses

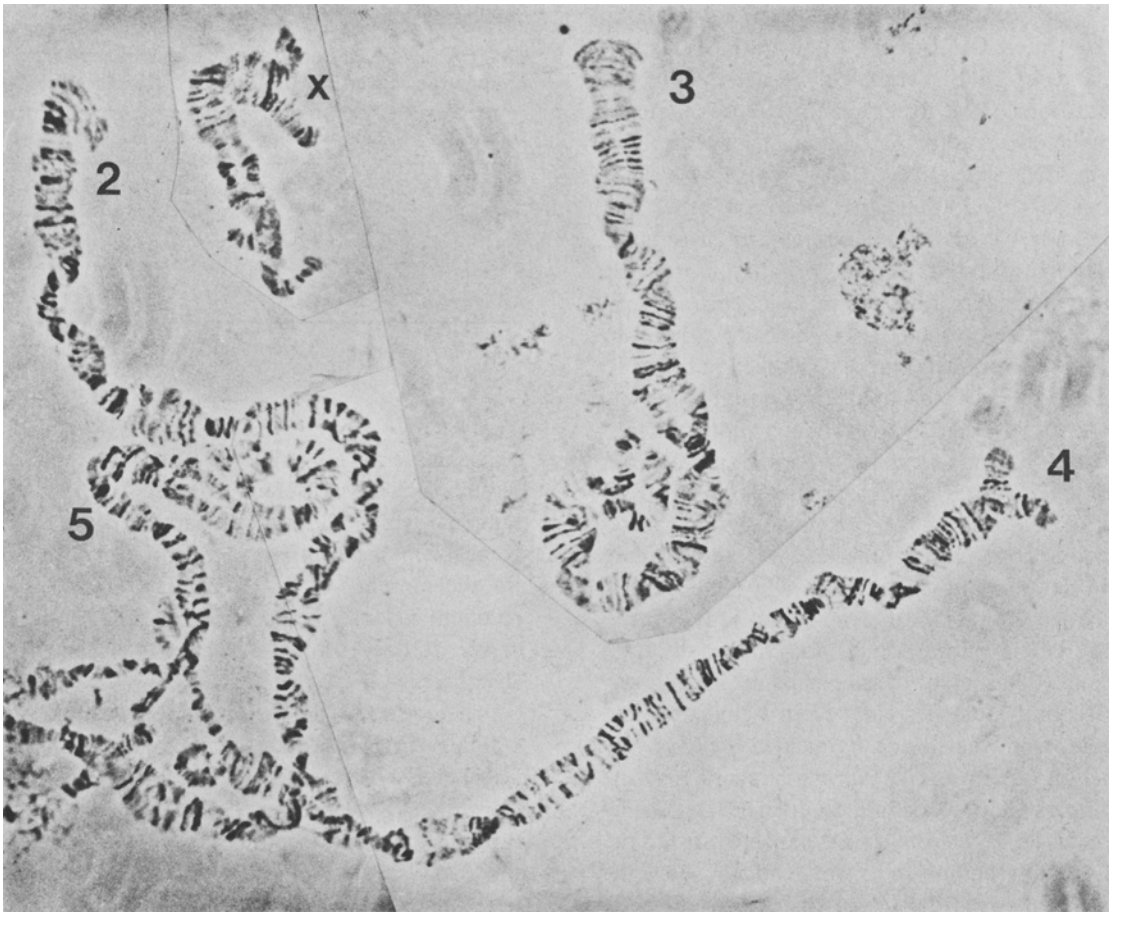

Fig. 2. A complement of ovarian polytene chromosomes from an F_1 female of a cross between *A. aruni*? and *A. funestus*. Note extensive asynapsis between apparently homologous band sequences.

generally. Neither parental species showed such asynapsis in our samples.

Discussion

The cytological distinctness of *A. parensis* and *A. funestus* permits unambiguous identification of adult females and should be useful in epidemiological studies where *funestus* populations are involved. However there is need for some caution with our results. We saw two insects from Kanyemba that were heterozygous for an inversion close to 3c, but poor quality of these slides did not permit accurate identification of the breakpoints. We did not score arm 2 in these specimens.

The fact that all three species share the same X arrangement is contrary to the expectations of Kitzmiller (1977). In reviewing anopheline cytology he makes the point that 'In most cases the X chromosomes are distinctive enough to permit species identification using only the X.' He does point out some exceptions but uses the *A. gambiae* group as an example of fixed inversion differences on the X marking species differences in *A. gambiae* (species A), *A. arabiensis* (species B) and *A. quadriannulatus* (species C), and ignores the fact, previously made by him, that three other species of the group share two of these X arrangements, *A. merus* and *A. gambiae* share one arrangement which differs by two inversions from the X shared by *A. quadriannulatus, A. melas*, and species D (as yet not formally named), which differs by a

further three inversions from *A. arabiensis*. We suspect that two different data sets might be responsible for the apparent correlation of highly distinctive Xs and species. The one set is the correlation of chromosome arrangements to formally-recognised, specific taxa, and the other represents chromosome arrangements of biological species which have been recognised from data other than morphological divergence, our suggestion being that formally-recognised, specific taxa in anophelines are highly suspect and may very often cover species complexes. The simple karyotyping of formal taxa might well represent one of several species within each taxon and a subsequent comparison of these chromosome arrangements would maximise the likelihood of 'species' differences. Another related question raised by Kitzmiller's views (1977) is, just how are 'species' recognised in anophelines, and what implications do homosequential species like *A. funestus* and *A. aruni*? have for such recognition?

Firstly we have alpha taxonomy; a pigeon-holing of individuals having similarity of appearance. Each pigeon hole is a subjective decision by a taxonomist and represents a 'species'. The importance and usefulness of this first stage in understanding any group of animals is in no way compromised in anophelines by the fact that we know the pigeon holes very often represent species groups rather than single biological species. Secondly perhaps our most sensitive means of species recognition results from detailed study of natural populations in relationship to disease transmission. Here very different behaviour patterns have been detected in populations that are referred to single pigeon holes in taxonomy (e.g. the *A. gambiae* complex, Paterson 1963; the *A. maculipennis* complex, Bates 1940). Subsequent study may find subtle morphological differences as in the cases of the *maculipennis* complex and the species reported here, correlated to the behavioural characteristics. Thirdly cross-breeding experiments may lead to species recognition when hybrid sterility occurs in interspecific hybrids. And finally study of chromosomal rearrangements may lead to species recognition (e.g. Stegnii & Kabanova 1978). For all practical purposes a species will come to be 'defined' in terms of the most efficient means of identification. For example, cytological study of the *gambiae* complex (Coluzzi & Sabatini, 1967, 1968, 1969) rests on a very small sampling of nature. This was no fault of the workers but a measure of the difficulty of the then current means of species identification, i.e. cross-breeding progeny from wild-caught females to known laboratory stocks and scoring resulting progeny for sterility/fertility (Davidson 1964). Very limited numbers of wild-caught individuals could be identified in this way. Once chromosomal differences were correlated with the mating types, together with Coluzzi's (1968) discovery of ovarian polytenes in anophelines, chromosomes came to be the sole means of identification of wild-caught material. The work of Mahon *et al.* (1976) and Miles (1978) on protein variation and species-specific electromorphs utilised chromosomally identified material and so did not effectively alter the fact that the *gambiae* species are chromosomally defined. If total dependence is placed on chromosome rearrangements as species markers then we are trapped into similar thinking as the typologist who equates the pigeon holes of alpha taxonomy with biological species.

Two workers have suggested that in anopheline speciation, chromosomal rearrangements may be a primary cause of speciation, thus giving weight to the idea that such rearrangements bear a causal relationship to speciation. It would follow from this idea that chromosomal rearrangements should be expected to be very good species markers. M.J.D. White (1973, p. 369) in discussing *A. gambiae* and *A. arabiensis* notes that their autosomes are very similar, but that the X chromosomes are 'profoundly different'. Later (pp. 401-402) he remarks 'There seems to be a strong suggestion that in a few groups (e.g. ... the *Anopheles gambiae* complex) speciation follows on situations where there are ... two different types of X chromosomes coexisting in an ancestoral population'. The exact situation about the X chromosomes, *A. gambiae*, and *A. arabiensis*, together with their close relatives is outlined above. It gives no suggestion whatsoever of rearrangements of the X chromosomes having a causative role in speciation within the group. G.B. White (1973) says of species D 'chromosome inversions apparently embody the genetical causes of its differentiation'. However, whilst species D does have a unique arrangement for the total genome, it does not possess a single unique inversion with respect to the other species in the *gambiae* complex (White 1974). Neither of these authors offer any convincing evidence that we should abandon the more orthodox view that chromosomal rearrangements, particularly

paracentric inversions, are incidental correlates of speciation.

The *funestus/aruni*? pair of species are the second reported example of homosequential species (*A. labranchiae/atroparvus*, Frizzi 1947, Coluzzi 1970) in anophelines. In both examples there are morphological differences, and the species have different behavioural characteristics. These examples should not be considered rare in reality, because we do not have the means of deciding whether they are 'rare' because of technical bias, or because they are actually 'rare' in nature. They are a warning that chromosomal rearrangement, like morphological differences, might or might not accompany speciation events.

That *A. funestus* and *A. aruni*? are homosequential raises a question about the work reported here. How were the samples identified as *A. funestus* and *A. aruni*?? The answer is that they were not directly identified. Firstly the holding period of one and a half days of wild-caught material until females are half gravid results in some damage to most insects. Scales are rubbed from both wings and palps, thus we could not measure the variables used by de Meillon *et al.* (1977) to discriminate between these species. We could not effectively use hybrid sterility because both species are difficult to maintain in the insectary, and, hence it is difficult to have both alive together in the laboratory. Indirectly we can use the levels of polymorphism of inversions. The rather limited samples of *A. aruni*? suggest that the species is monomorphic for $3+^{a}$, $3+^{b}$, and $5+^{a}$, whilst *A. funestus* maintains relative frequencies for these three inversions near 0.5 in southern African populations at least.

If large numbers of *A. aruni*? were mixed with *A. funestus* in our samples we would expect a deficiency of heterozygotes compared to expected genotypic frequencies derived from the Hardy-Weinberg equilibrium. Furthermore this would be most marked in subsamples of outdoor biting insects since *A. aruni*? appears to have a preference for outdoors. We will present details of such sub-samplings elsewhere, however we should mention that in fact such relative heterozygote deficiency was noted in outdoor samples from Kanyemba. However the distortion was an excess of homozygotes for 3ab and not $3+^{a}+^{b}$ that would be expected from a mixture of *A. funestus* and *A. aruni*? A further possible resolution of this problem of species identification might come from a clarification of the 2ad complex. We recorded 2d from Kanyemba prior to the discovery of *A. aruni*?, and subsequent resampling at Kanyemba was and is not possible due to the civil war in Zimbabwe. Certainly 2d did not occur in the Namibian sample.

The two types of second chromosome floating in *A. aruni*?, i.e. 2ad, and $2+^{a}+^{d}$ must at sometime have been linked by either of the two intermediate types, $2+^{a}$d, and $2a+^{d}$. The latter type does occur in *A. funestus*. Wallace (1959) has suggested that where three or more arrangements exist for the same segment of a chromosome then all but two of these will be eliminated by selection. This is because any favourable sequence of genes, protected from recombination by any inversion, and that interacts with other sequences to give heterozygote advantage, will be broken up by recombination in the various homozygotes that would occur where more than two chromosome arrangements coexist in a population. Wallace (1959) called this the 'triad' hypothesis. The simplest explanation of the *funestus/aruni*? 2ad situation is that 2a floated against $2+^{a}$ in the ancestor of these two species, and remained after the speciation events leading to them. In some isolate of *aruni*? 2a was fixed and 2d superimposed on it as a polymorphism. This isolate then returned into sympatry with the parental population in which 2a/+ still persisted. Initially there would have been three types of second chromosomes, i.e. 2ad, $2a+^{d}$, and $2+^{a}+^{d}$ in the population; eventually $2a+^{d}$ was eliminated. Notice that $2a+^{d}$ and $2+^{a}+^{d}$ will allow recombination for the segment covered by $+^{d}$, but that the two chromosomes will have different gene sequences for $+^{d}$ having been derived from the two alternative sequences of the more ancient inversion 2a. A more detailed geographic sampling of *aruni*? may reveal remnants of these two postulated isolates.

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