Chromosomal Differences Among Species of *Anopheles* Mosquitoes

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ABSTRACT. All of the anopheline species thus far studied have distinctive cytogenetic features which permit identification to species. In some cases the cytogenetic differences are the only sure methods of distinguishing among members of a complex of sibling species. Easily seen cytogenetic differences are found in the X-chromosomes and in the autosomes, which often contain characteristic and unique inversions. Many of the banding pattern similarities occur in distantly related species and may extend to subgeneric levels. Most thoroughly studied thus far include: *Anopheles maculipennis* (nearctic and palearctic); subgenus *Cellia*, including the *An. gambiae* complex and several other species groups; subgenus *Stethomyia*; and subgenus *Nyssorhynchus*.

The *Anopheles maculipennis* complex.

The cytogenetic approach to anopheline identification begins with the *Anopheles maculipennis* Meigen complex and with the classical morphological, ecological, physiological and hybridization studies of Falleroni, Missiroli, Hackett, Bates and their colleagues. These studies ultimately culminated in the recognition that the mosquito which had been called simply *"Anopheles maculipennis"* was in fact a group of five species; perfectly good biological, genetic and evolutionary taxa which, however, had failed to evolve consistent morphological differences. Subsequent studies have somewhat refined the picture but the complex remains an example of what evolutionists term sibling species.

Carlo Jucci, a biologist and geneticist far ahead of his time, realized the potential of the cytogenetic approach and interested Guido Frizzi in pursuing it in the *An. maculipennis* complex. The result was a series of papers published between 1947 and 1967 describing the larval salivary gland chromosomes of the complex and establishing beyond doubt that species which were difficult or impossible to separate using the usual morphological criteria, could nevertheless be easily identified using chromosomal banding patterns and inversions. These observations were later extended by Frizzi to *An. quadrimaculatus* Say and *An. aquasalis* Curry, a North American and South American species, respectively. For a review of the early work see Kitzmiller, *et al.*, 1967.

The anopheline species in North America which are considered to be *"maculipennis"* species on morphological grounds, *An. occidentalis* Dyar and Knab, *An. freeborni* Aitken, *An. earlei* Vargas and *An. aztecus* Hoffman have been studied by Kitzmiller and colleagues (1967). These species fit better the systematist's idea of a species; there is better morphological differentiation, the four species generally are allopatric and some ecological/physiological differences may

be observed. All four species have unique X-chromosomes with no easily understandable inversion relationships to each other nor to the palearctic species.

The autosomes, however, have clear and unmistakable similarities. The free and centromere ends of each of the four autosomal arms have bands that are obviously in the same pattern. The extent of the similarity varies from species to species. The internal portions of each arm have been subject to rearrangement, chiefly by paracentric inversions, and much of each arm can now be diagrammed in terms of a "standard" pattern plus subsequent inversions; included, overlapping and tandem. More inversions have taken place in 3R, the longest arm, which therefore contains the least amount of apparent similarity. The most conservative arm, 3L, has relatively few inversions and therefore the relationships among the species can be easily followed in this arm.

Our work with the North American fauna also involved the species *An. quadrimaculatus*, *An. atropos* Dyar and Knab, *An. walkeri* Theobald, *An. punctipennis* (Say), *An. crucians* Wiedemann and *An. bradleyi* King (Kitzmiller, 1976). Again the X-chromosome of each of these species is unique, quite sufficient to identify the species. The totally unexpected feature of these preparations was that the autosomes all looked more or less like the palearctic and nearctic *An. maculipennis* autosomes, although admittedly the similarities were more tenuous and the differences relatively more pronounced.

Hybridization studies further complicated this picture. Details will not be presented here but considerable cross-fertility exists between *An. atroparvus* Van Thiel and *An. freeborni* and between *An. atroparvus* and *An. quadrimaculatus* as well as among the nearctic species.

Other North American species.

On morphological grounds *An. quadrimaculatus*, *An. atropos* and *An. walkeri* superficially resemble the species of the *maculipennis* complex. The X-chromosomes are, as usual, distinctive. The autosomes, especially at the free and centromere ends have many regions similar to or identical with correspondingly placed regions in *An. atroparvus*, *An. freeborni* and *An. earlei*. 3L again is very much alike in all 3 species and very much like 3L in *An. atroparvus* (Kreutzer, *et al.*, 1969; Kitzmiller, *et al.*, 1974).

The situation became further complicated upon examination of *An. punctipennis*, *An. crucians* and *An. bradleyi*, mosquitoes with light and dark scaled wings, morphologically very distinct from the *maculipennis* complex (Kreutzer and Kitzmiller, 1971a, 1971b, 1972). By now the distinct X chromosomes were expected, but the fact that the autosomes were very similar to those of *An. atroparvus* and *An. freeborni* was confusing. Not only the centromere and free ends were alike but long stretches of the autosomes were identical band for band. We found no consistent differences between 3L of *An. freeborni* and 3L of *An. punctipennis*. The southern species *An. crucians* and *An. bradleyi* are obviously related to *An. punctipennis* morphologically; the autosomes also show many similarities.

The situation became thoroughly confused when several neotropical species were examined. *An. pseudopunctipennis* Theobald, *An. hectoris* Giaquinto-Mira, *An. punctimacula* Dyar and Knab, *An. neomaculipalpus* Curry and *An. vestitipennis* Dyar and Knab, by no stretch of the imagination to be considered *"maculipennis"* species, still showed autosomes similar to species previously studied. The X-chromosomes, as usual, were characteristic and could be used for positive identification (Kitzmiller, *et al.*, 1967).

Once sufficient data were available, a reassessment of the evidence indicated that what we were observing in the autosomes was not a *"maculipennis"* similarity but a similarity which extended beyond this complex to several species within the subgenus *Anopheles*, to which all the species which had been studied, belonged. With respect to the North American species, a synthesis of the chromosomal, the morphological and the crossing evidence indicates three groups of species; *An. freeborni*, *An. occidentalis*, *An. earlei* and *An. aztecus*, closely related among themselves and more distantly but clearly related to the palearctic *maculipennis* complex: *An. punctipennis*, *An. crucians* and *An. bradleyi* (and *An. georgianus* King which has not been studied cytologically) all of which are related but do not show any degree of close relationship with the *maculipennis* complex; and *An. quadrimaculatus*, *An. atropos* and *An. walkeri*, related among themselves, probably distantly related to the *maculipennis* complex, but not at all closely related to the *An. punctipennis* group of species.

Generalizations from the North American data.

Among these three North American groups 4 generalizations may be made: 1) the X-chromosomes are all distinctive and sufficient for cytotaxonomic identification, 2) the autosomes are all generally similar, especially at the free and centromere ends of each arm, 3) the interior of each arm has undergone rearrangements, especially by paracentric inversions. In some cases known inversions will account for the differences between species, in other cases additional inversions, not yet recovered are necessary to account for the differences. 4) Autosomal similarities are closest within groups, i.e.: within the *An. maculipennis*, *An. punctipennis* and *An. quadrimaculatus* groups. However, these autosomal similarities are also common in distantly related species and may represent a pattern common to as large a group as the subgenus *Anopheles*.

The *Anopheles gambiae* complex.

The classic case of the use of the cytogenetic approach has been in the *Anopheles gambiae* Giles complex in the subgenus *Cellia* Theobald. This has certainly been the most important development in mosquito genetics, indeed in malaria epidemiology, in the last decade. Although several investigators have participated, especially in the field application of the techniques, the extraordinary breakthrough has been due to the brilliant work of Mario Coluzzi, both in the field and in the laboratory. The familiar pre-chromosomal story will not be detailed here, but for years *Anopheles gambiae* was, to say the least, a puzzling problem. It was evidently an important vector of malaria in the entire Ethiopian zoogeographical region, yet distinct ecological and behavioral differences in different areas were unquestionably correlated with variability in disease transmission. The first breakup of the monolithic *An. gambiae*

came in the separation of the salt water forms *An. merus* Dönitz and *An. melas* (Theobald) from fresh water *An. gambiae* not only on physiological grounds but also on the basis of hybridization studies. Additional hybridization tests in the late 1950's and early 1960's designed to probe the genetic basis of resistance to insecticides, led to the discovery by Davidson (Davidson, 1956; Davidson and Jackson, 1962; Davidson, 1962, 1964a, 1964b) that fresh water *An. gambiae* was actually a complex of three species A, B and C, so that with the brackish water species *An. merus* and *An. melas*, 5 species were recognized from what had been previously considered only *An. gambiae, sensu lato*. Later a sixth species, D, was discovered (Hunt, 1972; Davidson & White, 1972; Davidson and Hunt 1973).

For years the identification of the sibling species of the *gambiae* complex was a matter of isolation of colonies, cross-mating with known species and assessment of fertility in the F_1 generation, often a matter of 30-40 days from beginning to end. It was a laborious process, but one which eventually blocked out the distribution of the sibling species of the complex.

In a monumental series of papers Coluzzi and Sabatini (1967, 1968, 1969) showed that all 5 species could be distinguished cytogenetically. Later, a sixth species was identified by cytogenetic methods.

Species A, B and C, from fresh water, may be identified using the X-chromosome. Each has a distinctive X. If species C is arbitrarily taken as the standard arrangement, then species A differs from it, in the X, by two fixed inversions (a and g), while species B has three other fixed inversions (b, c and d). The arrangement X ag is common to species A and *An. merus*. The standard arrangement X is shared by species C, *An. melas* and species D. The arrangement X bed is distinctive of species B only.

Further fixed inversions are found on chromosome 2R of *An. merus* and *An. melas*. The arrangement 2R op is distinctive of *An. merus* and differentiates this species from species A. The arrangement 2R m is typical of *An. melas* and serves to differentiate this species from both species C and species D. These two species can be separated by the fixed inversion a on 3L which is shared by species D and *An. melas*.

Various polymorphic inversions were also found particularly in species B and species A. Polymorphic inversions were also recorded in *An. melas*, species C and species D. Most of these polymorphic inversions are on chromosome 2R. However three of the 2R inversions are shared between species A and B and according to Coluzzi *et al.*, (1974) were transferred from one species to the other by introgressive hybridization.

Thus all six species of the *An. gambiae* complex may be clearly and definitively identified cytogenetically.

Other species and species complexes within the subgenus *Cellia* have been investigated. The two species of *An. farauti* Laveran in the *An. punctulatus* Dönitz complex have been separated on hybridization data and also show clear chromosomal differences (Bryan, 1973a, 1973b, 1973c; Bryan and Coluzzi, 1971). One important difference however, seems to be that the X chromosomes are seemingly identical but two fixed inversions are found in chromosome 2.

Cytological and taxonomic relationships within the subgenus *Cellia*.

Maps are also available for *An. superpictus* Grassi, *An. maculatus* Theobald, *An. stephensi* Liston, *An. pulcherrimus* Theobald, *An. tessellatus* Theobald and *An. subpictus* Grassi, also in the subgenus *Cellia*.

Apparently constituting one of the few exceptions to the uniqueness of the X-chromosomes, the X is apparently identical in *An. superpictus* and *An. stephensi*.

Anopheles superpictus and *An. stephensi* differ by 4 fixed inversions, one in 2R, two in 2L and one in 3L. Six additional fixed and floating inversions are found in different geographic strains of *An. stephensi* (Coluzzi *et al.*, 1970, 1973).

The other four *Cellia* species (excluding *An. farauti*) have readily distinguishable X chromosomes. The autosomal similarities, adduced by band for band comparisons (but no hybrids), appear to follow the taxonomic separation into series within the subgenus.

Series *Pyretophorus*. Both *An. gambiae* and *An. subpictus* belong to this series. The X of *An. subpictus* shows many similarities to that of *An. gambiae* species A and the autosomal arms are quite similar except for 3R, which is more like 3R of *An. maculatus* (Narang, *et al.*, 1973a).

Series *Neocellia*. Four species belonging to this series, *An. superpictus*, *An. stephensi*, *An. maculatus* (Narang, *et al.*, 1973b) and *An. pulcherrimus* (Baker *et al.*, 1968) have been mapped. The similarities of *An. superpictus* and *An. stephensi* have been discussed above. Many of the blocks of bands are similar in chromosome two in *An. maculatus* and *An. pulcherrimus* but at least 9 inversions must be involved to account for the differences. In chromosome three, these species also show similarities in blocks of bands but rather drastic chromosomal events including two interarm exchanges, a pericentric inversion and several paracentric inversions must have occurred to account for the present pattern. About 2/3 of the bands in the autosomes of *An. maculatus* appear to be similar to those in *An. stephensi*, with the most prominent similarities in chromosome 2. These two species, *An. maculatus* and *An. stephensi*, appear closest when the autosomal banding patterns are compared but are completely isolated genetically.

Series *Neomyzomyia*. Chromosome maps are available for three species of this group, *Anopheles tessellatus* (Narang, *et al.*, 1974) and two species of *Anopheles farauti* (Bryan and Coluzzi, 1971). The X of *An. tessellatus* is distinctive but the X is identical in both species of *An. farauti*. The autosomes do not show any apparent similarity to those of *An. maculatus*, *An. stephensi*, *An. pulcherrimus* or *An. subpictus*. There is some slight similarity in *An. tessellatus* with 2R of *An. gambiae*. Thus the chromosomes of *An. tessellatus* and *An. farauti*, belonging to the primitive *Neomyzomyia* series of *Cellia*, appear to be quite different than those of other series within this subgenus.

Chromosomal relationships among neotropical anophelines.

Stethomyia Theobald. This subgenus contains five species, all neotropical. They are separated with difficulty on minor morphological characters. Two species, *An. nimbus* (Theobald) and *An. kompi* Edwards, have been mapped (Kitzmiller, *et al.*, 1977). The X-chromosomes are very different, one about twice the length of the other. The autosomes are surprisingly different for two species which are evidently so closely related. Only about 2/3 of the larvae of these two species may be identified using the cited morphological characters; 150 out of 151 in one sample were correctly identified chromosomally.

Nyssorhynchus Blanchard. This neotropical subgenus has a wide distribution from Texas and Florida, through Mexico, Central America, most of South America and the Caribbean. Its 24 species include most of the important neotropical malaria vectors.

It is also a very "difficult" subgenus from the taxonomic point of view. The characters used for identification include minor variations in chaetotaxy and coloration, subject to wide variation geographically and environmentally. The distinguishing characters are inconsistent over the range of a species and are evidently subject to genetic variations as well. For example, eggs from a single female of *An. nuneztovari* Gabaldon can produce larvae which may be identified as 4 species; these same larvae may produce adults which can be classified as three species! The situation is complicated by the fact that sibling species evidently exist in the common, widespread and medically important species. There is evidence of cytotaxonomic speciation in at least 3 species, *An. nuneztovari, An. darlingi* Root and *An. albitarsis* Lynch Arribalzaga.

Fortunately, all species studied so far have good to excellent larval salivary chromosomes, permitting construction of maps and the study of inversions. Nurse cell chromosomes have so far been uniformly poor.

All species thus far studied have distinctive X-chromosomes. The autosomes are all very much alike, especially at the free and centromere ends of each arm, and often for long stretches in the center. Most of the variation in banding pattern within each autosomal arm has evidently been due to paracentric inversions.

Anopheles nuneztovari is distributed from Colombia and Venezuela through the Amazon basin, covering most of Brazil. In most of its range it is not considered to be an important vector, but appears to be responsible for transmission in many areas of Colombia and Venezuela. The Colombian and Venezuelan populations differ from the Brazilian ones by a fixed inversion in the long arm of the X (Kitzmiller, *et al.*, 1973). Hybridization between the two populations has so far not given positive results.

Another widespread species, *Anopheles albitarsis*, is locally very abundant and although not considered to be a primary vector is often associated, in large numbers, with outbreaks of malaria. Three chromosomal types of *An. albitarsis* have been identified -- two in southern and eastern Brasil, a third in Colombia and Venezuela. All three differ by fixed inversions in the X-chromosome and by a series of fixed and floating inversions in the autosomes. Two of these types occur sympatrically but evidently do not interbreed since critical inversion heterozygotes appear to be lacking. Only two of 22 inversions are shared between

the B_1 and C populations; the B_2 population so far has 12 inversions, none shared with B_1 or C (Kreutzer, *et al.*, 1977).

Generalizations from the cytogenetic data.

There are now sufficient data to permit some generalizations concerning the cytogenetic differences among species, which in at least a restricted sense are reducible to cytotaxonomy.

1) In most cases the X chromosomes are distinctive enough to permit species identification using only the X. Polytene chromosomes vary with respect to the position of the centromere and hence may be classified as metacentric, submetacentric, subtelocentric, etc. This pattern in the polytene chromosomes may or may not correspond to the picture in the mitotic sex chromosomes in which the size and shape are influenced by the amount of heterochromatin present. The polytene X-chromosomes usually show, in addition, a distinctive banding pattern which will unmistakably identify the species. There are certain exceptions to this generalization of distinctive X-chromosomes. These exceptions permit the construction of a sequence in the order of degree of identity of the banding patterns of the X-chromosomes. The most obvious exceptions are *Anopheles farauti* No. 1 and No. 2 in which the X chromosomes are identical and *Anopheles stephensi* and *Anopheles superpictus,* which also have identical X-chromosomes. An intermediate type is one in which the X differs in the frequencies of floating inversions, so that the same banding pattern is theoretically possible but rarely if ever occurs because of the high frequency of one or more arrangements. This type of situation is found in the cases of *Anopheles punctipennis* and *Anopheles perplexens* and between *Anopheles crucians* and *Anopheles bradleyi.*

A third level of differentiation is that in which X-chromosomes differ by fixed inversions and thus present clearly different banding pattern sequences. There are many instance of this type, the best known being the three fresh water species A, B and C of the *An. gambiae* complex, and the Brazilian and Colombian/Venezuelan populations of *An. nuneztovari.* The banding patterns are distinctive but can be easily derived by marking the fixed inversion sequences. Finally there is the large majority of cases thus far studied in which the X presents a distinctive banding pattern and/or a distinctive arm-length ratio, so that the X is immediately diagnostic. The species of the subgenus *Nyssorhynchus* fall into this category, as do most of the species of the subgenera *Anopheles* and *Cellia.*

Why should the X-chromosome, during chromosomal evolution, have been so sensitive to rearrangements? The phenomenon is not restricted to *Anopheles* but is also found in many groups of *Drosophila.* In the cases in *Drosophila* where it has been studied, the rearrangements have essentially been due to many, generally small, paracentric inversions, the same type of pattern found in the autosomes of the anophelines. Given the generally smaller lengths of the polytene X chromosomes and the not too frequent floating inversions in X-chromosomes in current populations, the evolution of the pattern of the X remains puzzling. One possibility is that inversions tend to become fixed in the X chromosome quite readily although I know of no mechanism which accounts for this. Another possibility is that the present pattern of the X chromosome is a relict of a former evolutionary period during which rapid and frequent

inversions in the X became fixed, perhaps in periods of rapid adaptation to a variety of environments. As more data become available on the adaptive significance of inversions we might hope to be able to understand this phenomenon more fully.

2) Autosomal patterns follow phylogenetic relationships. Species which, on classical taxonomic grounds are considered to be closely related, tend to have autosomal patterns which are alike. This is nothing new or unexpected - it occurs commonly in *Drosophila*. What might be unusual is the extent and breadth with which this occurs, perhaps even to subgeneric levels. In related species the autosomes tend to have similar banding patterns, especially at the free and centromere ends of the arms. Most of the interior of each arm has been rearranged by paracentric inversions. With careful study differences between species may often be charted with reference to these inversions.

Considerable variation occurs within this framework. *Anopheles labranchiae* Falleroni and *Anopheles atroparvus* are essentially homosequential species. The other members of the palearctic *An. maculipennis* complex differ only in a few third chromosome inversions.

Anopheles superpictus and *Anopheles stephensi* are closely related and the banding pattern of *An. stephensi* may be expressed in terms of the standard formula of *An. superpictus*. Furthermore, different geographic strains of *An. stephensi* differ by inversions from the standard strain and formula.

In the *An. gambiae* complex some species are conservative - not much rearrangement from species C, arbitrarily chosen as standard, yet species B shows considerable variability in banding pattern, depending upon which of many autosomal inversions are present.

Different species groups exhibit different rearrangements in given chromosomes. In the palearctic *An. maculipennis* complex, all known autosomal inversions are in chromosome 3, none has been found in chromosome 2. In the North American species, chromosome 2 inversions are frequent. The left arm of chromosome 3 is conservative in North American species with few inversions, and these usually of considerable length, within the arm. This conservatism extends beyond the *An. maculipennis* complex - for example *An. punctipennis* and *An. freeborni* have identical banding patterns in 3L (homosequential) yet these arms are completely asynaptic in hybrids.

Within the subgenus *Anopheles* traces of the "primordial" pattern may be found even in widely separated species such as *An. hectoris, An. pseudopunctipennis, An. neomaculipalpus, An. vestitipennis, An. claviger* (Meigen), *An. plumbeus* Stephens, *An. sinensis* Wiedemann and *An. sineroides* Yamada. These "homologies" are strongest at the free and centromere ends of the arm, but blocks of bands in the middle of some arms appeared to have retained their identities during chromosomal evolution, even with considerable divergence. It is tempting to ascribe "adaptive significance" to these "supergenes" which have survived considerable amounts of inversion polymorphism.

Similar autosomal patterns are found within the subgenus *Nyssorhynchus*. Most free and centromere ends of autosomal arms are quite alike, and the degree of rearrangements of the internal portion of each arm varies with the species.

3) Cytotaxonomic "total identification" is now possible for most species. A first approximation may be made using X-chromosomes, which will positively identify most species. In those cases in which an X-chromosome is not sufficient, then characteristic autosomal arrangements will give conclusive results. The *gambiae* case is an excellent example. An exactly parallel situation exists in the three cytospecies of *Anopheles albitarsis*.

4) Sooner or later we must be prepared to prepare keys based on polytene chromosomes. This at first presupposes a small group of elite practitioners, but sooner or later working entomologists must be trained to make and read slides.

5) The day is not far away when cytospecies will be described, and named, and put into museums. After all, chromosome morphology is just another kind of morphology but before this type of taxonomy obtains wide approval many precautions, preparations and investigations must be pursued. Probably for several years the cytospecies will be at best afforded discussion under "species groups" in taxonomic treatises.

6) We must not expect cytogenetics to be the "final answer". We shall be able to do several practical things: 1) identify the members of sibling species complexes, 2) obtain basic evolutionary data on phylogeny and systematics, 3) correlate cytogenetics and adaptation. But we shall not, for example, be able to rely on cytogenetics in all cases. Consider the case of homosequential species, which will have to be separated on other grounds.

7) Finally we must keep in mind that the anophelines are somewhat different with respect to the usual parameters of evolution, both within the group and in contrast to the culicine and aedine mosquitoes. Generally speaking, morphological mutants, "markers", are rare in the anophelines as compared to other groups, and within the genus some species appear to have more mutants than others. Chromosomal mutations are numerous in some species, of moderate abundance in others, apparently rare in a few. Yet the anophelines have indeed evolved, biologically and genetically just as any other organism -- they differ in physiology, in ecological requirements, in their ability to transmit disease, in reproductive isolation. In other words they are good biological species in every sense of the word, except that they have given us few morphological handles to grasp. Rather they force us to delve into the physiological, ecological, behavioral and reproductive differences which after all, are the real mechanisms of evolutionary dynamics.

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