CHROMOSOMAL SIMILARITY BETWEEN *ANOPHELES PERPLEXENS* AND *ANOPHELES PUNCTIPENNIS*

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INTRODUCTION. *Anopheles perplexens* Ludlow 1907 has a widespread but apparently spotty distribution in the eastern and southern United States. This mosquito is closely related to *Anopheles punctipennis*, from which it differs chiefly in its darker color, reduction of the costal spot, and fewer branches on hair two of larval segments 4 and 5. These and other morphological, physiological and ecological differences are fully discussed by Bellamy (1956) who proposed that it be accorded specific status rather than be considered merely a melanistic variant of *punctipennis*.

An examination of the salivary gland chromosomes of anophelines in other species has shown that closely related species and even sibling species may sometimes be distinguished on the basis of the banding patterns of these chromosomes (Coluzzi and Sabatini 1967, Kitzmiller, *et al.* 1967). The development of a method for induced copulation (McDaniel and Horsfall, 1957) permitted crosses between species which otherwise might not cross either in nature or in the laboratory. Using induced copulation it has been possible to cross *punctipennis* and *perplexens* in both directions, estimate the amount of fertility and genetic affinity between them, and study the appearance of F_1 hybrid chromosomes. The present paper describes the chromosomes of *perplexens* and compares them with those of *punctipennis*. The hybridization and F_1 chromosomal data will be reported elsewhere.

MATERIALS AND METHODS. The specimens from which this map was made were collected about 5 miles west of Cordele, Crisp County, Georgia. An abandoned mill, a concrete culvert and a concrete tunnel under the mill were the favorite resting sites of large numbers of *perplexens* adults. About 80 percent of the females collected were *perplexens*, about 20 percent were *quadrimaculatus*. No *punctipennis* or *crucians* adults were taken, although *perplexens*, *quadrimaculatus* and *crucians* larvae were collected from the mill pond and from quiet backwaters off the mill stream. Collections were made in March, April, June, September, October, November and December, from 1966 to 1970.

A. perplexens has been maintained in the laboratory by artificial copulation similar to the method described by Baker, *et al.*, 1962. In addition to the morphological differences between the two species certain behavioral differences associated with copulation were noted. The *punctipennis* males grasp the females with their claspers and almost immediately begin the pumping motion associated with insemination, but *perplexens* males and females remain clasped together for a longer period before the initiation of the pumping motion. The pumping motion is of short duration in *punctipennis* as compared to *perplexens* in which it continues for a longer period of time.

It has been very difficult to rear *punctipennis* in the laboratory, and there has been no long term colonization of this species. On the other hand *perplexens* is very easily colonized, and has been maintained by artificial copulation since it was first collected in 1966.

Chromosomes were prepared according to the standard method for anophelines (French, *et al.*, 1962). Dry ice was used to freeze the tissue prior to removal of the siliconized cover slip, and the preparations mounted using Zeiss Einschlussmittel L-15. All observations were carried out with a Zeiss phase contrast system.

DESCRIPTION OF THE CHROMOSOMES. The

salivary chromosomes of *Anopheles perplexens* are, superficially, and in most details, similar to those of *Anopheles punctipennis*. (Figure 1). They consist of a short X-chromosome (63μ) and two pairs of autosomes, one with arms of approximately equal lengths (2R, 150μ; 2L, 135μ) and one with arms of unequal lengths (3R, 215μ; 3L, 100μ). The chromosomes do not differ appreciably in gross morphology from those of *punctipennis* and therefore the numbered and lettered zones follow those of *punctipennis* whenever possible. As in *punctipennis*, the X contains zones 1–5; 2R, zones 6–14; 2L, zones 15–21; 3R, zones 22–32; and 3L, zones 33–39. For further description of the karyotype of *punctipennis* see Baker and Kitzmiller, (1964).

X-CHROMOSOME. The short X-chromosome presents a banding pattern very similar to that of *punctipennis* (Figure 2). At least two and possibly three paracentric inversions occur in the natural population of *perplexens*, and therefore at least four major banding arrangements are possible, eight if a minor 3 or 4 band inversion near the centromere (5C) is taken into account. One of these inversions is a long

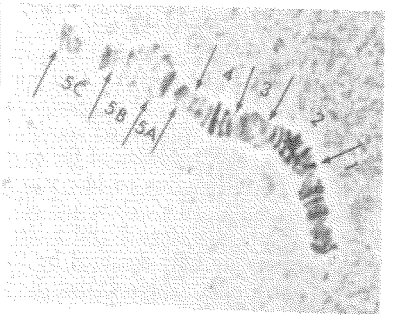

FIG. 2.—The most common banding sequence in the X-chromosome.

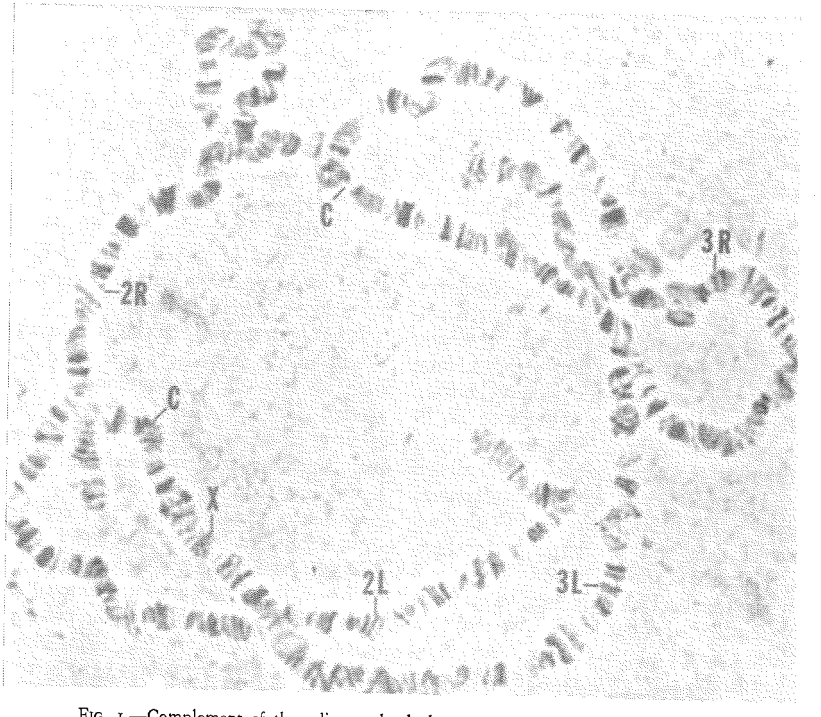

FIG. 1.—Complement of the salivary gland chromosomes of *Anopheles perplexens*.

one (1C to 4C) and involves the entire middle of the chromosome. The second is closer to the centromere, (5A–5B) and involves an area which, even in homozygotes, appears puffed, weakly banded and indistinctly stained. The arrangement most common in our slides, which admittedly are made from a laboratory population which has been colonized from a small sample of a single natural population, is described below and is figured in the map (Figure 3). The numbered and lettered zones correspond in so far as possible to those of *punctipennis*, but the *perplexens* map is considerably more detailed, and therefore the zones do not exactly coincide.

The free end of the arm consists mostly of light bands in 1A. This region is often flared, and the bands are often broken. In 1B are two thin dark bands, one at the beginning and one at the end, characteristic of the free end of the chromosome. At the end of 1B is one break point for a long inversion. At the beginning of 1C are three bands of medium intensity, followed in 2A by a sequence of three dark bands, the middle one of which is lighter. The two dark bands in 2B are always heavily stained, and followed in 2C by three bands which stained somewhat more lightly. This sequence of 5 bands is a good recognition area for the free end of the chromosome. Another usually consistent feature is the light puff in 2D, 3A and 3B. This area is lightly staining, expanded, and contains 4 dark bands, usually dotted, at one end. A prominent series of dark bands in an expanded area is found in 3C, 4A, 4B and 4C. This series of evenly spaced dark bands of which the one at the beginning of 4B is thickest and most heavily staining, is an excellent landmark. The narrow constriction in 5A marks the end of the long inversion which begins in 1C. The proximal end of the chromosome is characteristically widely expanded, asynaptic, mostly weakly staining and difficult to follow. An inversion takes up most of this area, from 5A through 5B, and when in the heterozygous state, the contained bands are so weakly stained and irregular that accurate identification of bands is very difficult. At the beginning of 5A are two bands of medium intensity, followed by two light bands. At the beginning of 5B is a double dark band, the most consistent landmark of the area. At the center of 5B are two irregular double bands, usually broken and lightly-stained followed by a pair of light bands and a thin band of medium intensity, usually sharp. A group of three light dotted bands marks the end of 5B, and also the end of the 5A–5B inversion. Nearest the centromere in 5C are mostly weakly staining bands with three, usually in a 1–2 pattern, the most prominent.

CHROMOSOME 2, RIGHT ARM. In all specimens of *perplexens* thus far studied the free end up to region 7D and the centromere end starting at 11B of 2R are almost identical with *punctipennis*, but the center section of the arm in *perplexens* is quite different from *punctipennis*. This area in *perplexens* has been involved in two paracentric inversions. One extends from 7D through 10A of the *punctipennis* arrangement and in *perplexens*, 8A through 10A. This section is one which is inverted in an aberration found in the natural population of *punctipennis*. A second shorter inversion extends from 10B to 11B of *punctipennis* and 10B to 11B in *perplexens*, and has a common break point with the longer inversion at 10A. The derivation of *perplexens* 2R from *punctipennis* 2R is diagrammed in figure 4. No inversions have been found in this arm in the natural population.

The best place to begin study of 2R is with the two groups of dark bands in 8B and 8D. Regions 10C and 11A have slight differences in band intensities from *punctipennis*. In 13C the three dark bands are differently spaced than in *punctipennis* so that the last one stands somewhat apart from the first two. Region 14 is often twisted and may appear asynaptic. The principal differences in this arm between the two species are the inversions and some staining intensities. The inverted regions

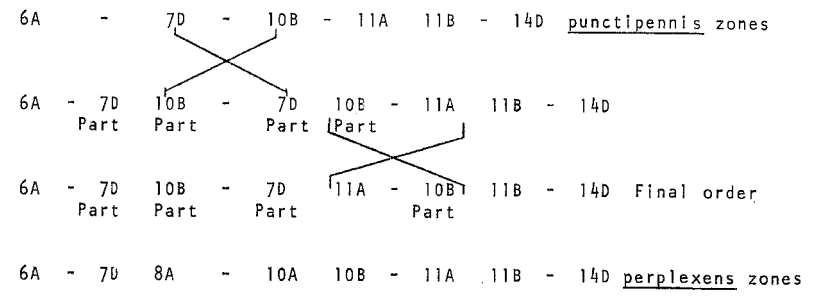

FIG. 4.—2R, *perplexens*. Two paracentric inversions necessary to derive the *perplexens* arrangement from that of *punctipennis*. Numbers and letters are those of the *punctipennis* map.

have the same but reversed patterns in both species.

CHROMOSOME 2, LEFT ARM. 2L in *perplexens* does not differ except in band intensity from 2L of *punctipennis*. No inversions have been found. The principal recognition areas are the single heavy band at the beginning of 21C flanked by light areas, the heavy puff in 20C, a series of heavy bands usually on either side of a constricted area in 18C, 18D and 17A, a long puff with dark bands in 16A, 16B and 16C, and the heavy bands as shown in region 15.

CHROMOSOME 3, RIGHT ARM. The right arm of chromosome three in *perplexens* is almost exactly the same as in *punctipennis* with the differences in the banding pattern due almost entirely to paracentric inversions. At the free end, the pattern is the same from 22A through 25B. At the centromere end 31A through 32D is identical in both species. The middle part of the arm has been involved in at least two paracentric inversions. One of these involves break points at 26A and at 30D, so that 30D–29C now follows 25D, near the free end of the arm, and most of 26A and 26B are now found toward the centromere end of the arm. Next, a long included paracentric inversion with breakpoints at 26C and 29B reinverts this section, so that the final arrangement, in terms of *punctipennis* zones is as follows: 22A–25D 30D–29C 26C–29B 26B–26A 31A–32D.

The inversions required to produce this arrangement are diagrammed in Figure 5.

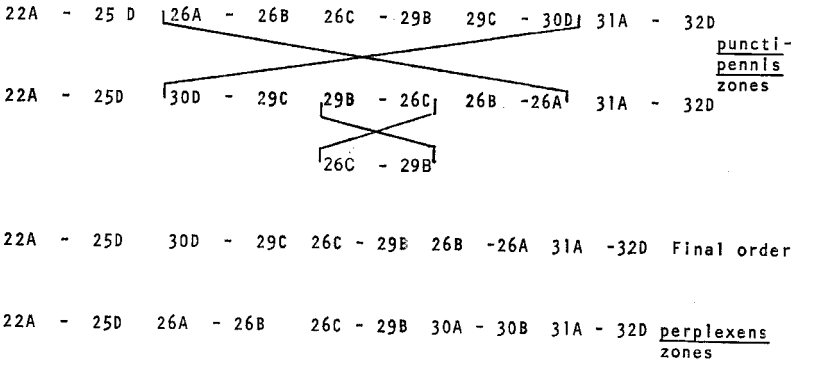

FIG. 5.—3R, *perplexens*. Two paracentric inversions necessary to derive the *perplexens* arrangement from that of *punctipennis*. Numbers and letters are those of the *punctipennis* map.

The similarities in this long arm are quite remarkable between the two species. Only in two areas, 25C–26B and 30A–30B do the maps fail to compare very closely. Close examination of these areas in slides of both *punctipennis* and *perplexens* clearly shows that the present *perplexens* map is more accurate in these areas than is the original *punctipennis* map. The areas involved are as shown on the present *perplexens* map. Other than these differences, the only differences in banding pattern are in staining intensity, or the occasional addition or deletion of a single band.

The principal recognition areas of this arm are the flared tip with 2 dark bands in 22A, the three small puffs in 22B, 22C and 23A, the heavily banded area in 25C and 35D, the sequence of bands in 26A and 26B, the three light but consistent puffs in 27D, 28A and 28B, the dark sequence in 28C, and the typical banding pattern from 31A to the centromere.

CHROMOSOME 3, LEFT ARM. The left arm of chromosome 3 does not differ in any major respect from 3L of *punctipennis* and *freeborni*. A few bands in some areas appear more distinct, probably as the result of better slides and improved optical equipment; a few bands differ in intensity. This arm is nevertheless, another "conservative" 3L, which so far is typical of the species within the subgenus *Anopheles*. For a detailed description of this arm see Kitzmiller and Baker (1963) and Baker and Kitzmiller (1964). Following are the principal differences between *perplexens* and *punctipennis* (or *freeborni*). In 39B and 39C the spacing and intensities of the bands are slightly different; in 39E the three heavy bands in the *punctipennis* map are replaced by four bands of lighter intensities; in 38A the second heavy band is now lighter; in 36A the last heavy band in the series of four is of lighter intensity.

INVERSIONS. Three inversions occur in this population sample, two in the X and one in 3R. A third, small inversion in 5C in the X involves only three bands and occurs with a low frequency. The most common arrangement of the X is shown on the map. The next most frequent arrangement is homozygous for both inversion A and inversion B at the same time, a most unusual situation. (Fig. 6). In-

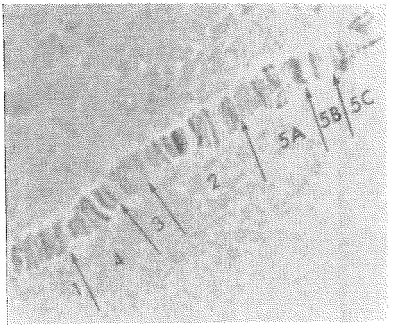

FIG. 6.—Second banding sequence found in the X-chromosome.

version A alone has not been recovered in the homozygous condition, nor have homozygotes for inversion B alone been recovered. The only X-chromosome aberration recovered is heterozygous for both inversions. (Fig. 7). The aberration is diagrammed in Figure 8.

Two relatively simple events will derive the *perplexens* 3R arrangement from that of *punctipennis*. A long paracentric inversion, involving more than half the length of the arm, 26A through 30D, appears to have been the primary event, and an included inversion 26C–29B, reinverts this section to the original order. Thus 29C–30D are now found in an inverted order near the free end of the arm and 26A–26B, also in the inverted order, near the centromere end of the arm. Zones 26A and 26B of the *perplexens* map are in fact zones 30D–29C of the *punctipennis* map. 26A is drawn considerably different from its original *punctipennis* counterpart, 30B–30D. The banding arrangement shown in the *perplexens* map is indeed present in the slides, and a recheck of the *punctipennis* slides clearly shows that this pattern is more accurate than that shown on the *punctipennis* map. Similarly 30A and 30B of *perplexens,* the inverted 26A–26B of *punctipennis,* has also

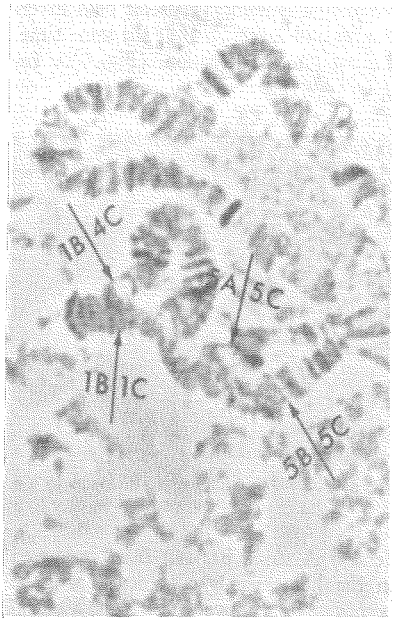

FIG. 7.—Inversion heterozygote in the X-chromosome found in the natural population. This organism received one normal X chromosome and one which contained both inversions.

been redrawn, and its greater accuracy checked against *punctipennis* slides.

As noted in other studies of closely related species (Kreutzer, *et al.* 1970) the areas near the breakpoints of inversions appear weak, of variable staining intensity, and are often asynaptic. This is especially true in the first part of 26A and the last part of 26B, the part of 30A nearest the

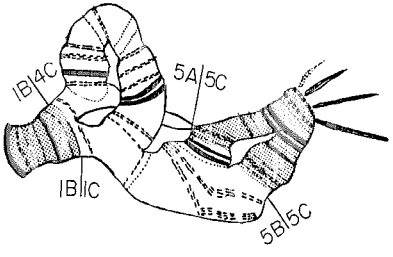

FIG. 8.—Diagram of the X chromosome aberration. Stipled sections represent the normal X sequence.

inversion, and the last part of 30B, nearest 31A. It is possible that these "weak points" are preferred sites of chromosome breakage. This is also true of the breakpoint areas for the inversions in 2R which are at the end of 7D, 8C and 11A.

Thus far the 26C–29B inversion is the only one found in the natural population of *perplexens*. (Fig. 9). Both arrange-

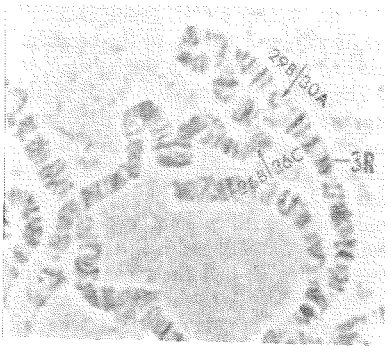

FIG. 9.—The 3R aberration. Numbers refer to *perplexens* map.

ments evidently exist in the population as evidenced from the heterozygotes, but only the arrangement shown on the map has been found homozygous. The chromosome which produces this aberration does not have the center section of the original inversion (26C–29B) inverted. The 26A–30D inversion is identical with one found in *punctipennis* (Baker and Kitzmiller, 1964). The other inversions described from 3R of *punctipennis* have not been recovered as yet from *perplexens*, but the 26C–29B inversion is very close to the 26C–28C inversion in *punctipennis* and shows a common break point at 26C.

DISCUSSION. The most striking feature of the banding pattern of *perplexens* is its similarity to that of *punctipennis*. The X chromosome shows the most difference, as is usually the case in the anophelines, but the autosomes are remarkably similar. Only in 2R and in 3R have relatively simple rearrangements taken place, due to presumed paracentric inversions. The 2R 7D–9C inversion as well as the 3R 26A–

30D inversion of *perplexens* are found in the natural population of *punctipennis*. The other two of the four autosomal arms are almost exactly the same in both species.

Similarity of banding pattern, and the presence of an identical inversion certainly argue for very close relationship between these two species. However, similarity of pattern alone does not necessarily mean close genetic affinity; this can only be tested by hybridization, fertility and the degree of synapsis of F_1 chromosomes.

The chromosomal differences parallel closely the morphological ones. In themselves, these differences are slight and hardly conclusive in judging the "validity" of these two species. If the 2R and 3R inversion differences turn out to be uniform in all collections of *punctipennis* and *perplexens,* it might be possible to use them as a taxonomic character. If not, the autosomes cannot be used. The X-chromosome is most different from that of *punctipennis* and may be reliably used to separate the species. The chromosomal data therefore support (1) the consideration of these two species as very closely related and (2) the separation of *perplexens* as a valid species from *punctipennis* on the basis of the distinctive pattern of the X-chromosome.

SUMMARY. The salivary gland chromosome map of *Anopheles perplexens* is described and compared with that of *Anopheles punctipennis*. The maps of the two species are very similar; however, there are differences between the two banding patterns in the X chromosome, 2R, and 3R. The chromosome pattern differences are caused by two or possibly three paracentric inversions, and the differences in 2R and 3R are also derived from paracentric inversions, two in each arm. These inverted sections have the same but reversed banding patterns in both species. Both 2L and 3L show minor band intensity differences. Two inversions found in the natural population of *punctipennis* as heterozygotes are homozygous in the *perplexens* arrangement. These studies indicate that these two species are chromosomally very similar, and were probably derived one from the other or separated recently from some common ancestor.

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