

# Cladistic analysis of polytene chromosome rearrangements in anopheline mosquitoes, subgenus *Cellia*, series *Neocellia*

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New data on rearrangements of ovarian polytene chromosomes from mosquitoes are presented for the following species in the series *Neocellia* of *Anopheles* (*Cellia*): *annularis*, *philippinensis*, *nivipes*, *splendidus*, *jamesii*, and *ramsayi*. Rearrangement has involved fixation of alternatives for paracentric inversions. Two fixed inversion differences allow for the first time the direct identification of wild-caught females of *philippinensis* and *nivipes*. There is a suggestion that the latter nominal species may include two genetic species. These data and those published for close relatives are drawn together in a cladistic analysis to suggest relationships between the species. The data are limited and total relationships are not clear within the group.

*Key words*: cladistic analysis, polytene chromosomes, *Anopheles*, mosquito, speciation.

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On présente des nouvelles données sur les réarrangements des chromosomes polytènes d'ovaires de moustique pour les espèces suivantes de la série *Neocellia* de *Anopheles* (*Cellia*): *annularis*, *philippinensis*, *nivipes*, *splendidus*, *jamesii* et *ramsayi*. Le réarrangement a impliqué la fixation d'alternatives pour les inversions polycentriques. Deux différences d'inversion fixées permettent pour la première fois l'identification directe de femelles capturées à l'état sauvage de *philippinensis* et de *nivipes*. Suggestion est faite que cette dernière espèce nominale comprend peut-être deux espèces génétiques. Ces données et celles qui ont été publiées pour les parents proches ont été comparées dans le cadre d'une analyse phylogénique visant à mettre en évidence les relations interspécifiques. Les données sont limitées et l'ensemble des relations n'est pas clair à l'intérieur du groupe.

*Mots clés*: analyse phylogénique, chromosomes polytènes, *Anopheles*, moustique, spéciation.

[Traduit par le journal]

## Introduction

The subgenus *Cellia* of anopheline mosquitoes is divided into informal groups or series of species of which *Neocellia* is one. There are about 20 species in *Neocellia*, most of which are confined to the Oriental Zoogeographic region, with five in the Afrotropical region and four in the Palaearctic and Mediterranean regions (Gillies and de Meillon 1968; Reid 1968). There are several very important vectors of human plasmodia in the oriental and mediterranean members of the group.

Interspecific relationships judged from the rearrangement of polytene chromosomes from the ovaries of adults have been published for *stephensi* Liston and *superpictus* Grassi (Coluzzi *et al.* 1970); *maculatus* Theobald, *pulcherrimus* Theobald, *maculipalpis* Giles, *rufipes* (Gough), and *pretoriensis* (Theobald) (Green 1982a). Furthermore, some members of *Neocellia* were used by Green (1982b) for out-group comparison in an attempt to apply cladistic principles for the analysis of polytene data from species belonging to *Anopheles* (*Cellia*) series *Myzomyia*. An argument was presented to justify the use of cladistics in analysis of polytene chromosomal data (following Farris 1978), and the limitations of these principles are shown for

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data sets of the kind presented by polytene chromosomes when no extrinsic evidence exists for an evolutionary direction from ancestor to derived species (Green 1982b).

Here we present data from the ovarian polytene chromosomes of wild-caught females belonging to the following species: *annularis* Van der Wulp, *philippinensis* Ludlow, *nivipes* (Theobald), *splendidus* Koidzumi, *jamesii* Theobald, and *ramsayi* Covell. Together with the published data, cladistic principles are used in an attempt to show interspecific relationships within Neocellia.

### Materials and methods

Adult females were caught biting man and animals as follows: *Anopheles annularis*,  $N$  (no. of insects) = 25, Pingtung Country, Taiwan, 22°10' N, 120°50' E;  $N$  = 27, Wawa Dam and  $N$  = 7, San Jose del Monte, Rizal, both near Manila, Philippines;  $N$  = 109, Mae Hong Son, Thailand, 19°12' N, 95°54' E;  $N$  = 5, near Chiang Kham, Thailand, 19°29' N, 100°21' E; and some material came from a colony in the Ross Institute (now extinct) originating from Bangladesh. *Anopheles philippinensis*,  $N$  = 2, Wawa Dam as above;  $N$  = 2 near Chiang Kham as above;  $N$  = 14, Ban Hin Tan, Nakhon Nayok Province; and a further  $N$  = 26 near Nakhon Nayok, 14°19' N, 101°18' E, Thailand; some material came from the colony housed in the Department of Medical Entomology, United States Component to the Armed Forces Institute for Medical Sciences (AFRIMS), Bangkok, Thailand, originating from Klaeng, Rayong Province, Thailand. *Anopheles nivipes*,  $N$  = 85, Ban Mae Hoi, 18°24' N, 98°36' E;  $N$  = 41, near Rong Kwang, 18°26' N, 100°24' E;  $N$  = 19, Khonburi, 14°32' N, 102°16' E;  $N$  = 3, Ban Nam Tone, 14°42' N, 100°51' E;  $N$  = 16 near Chiang Kham as above;  $N$  = 11, Mae Hong Son as above;  $N$  = 2, Ban Takian Duan, 14°20' N, 99°00' E;  $N$  = 8, Chiang Mae, 18°43' N, 98°57' E; all localities in Thailand. Chromosomally identified material from Rong Kwang was returned to AFRIMS for colonization and subsequently identified as *nivipes* from the diagnostic pupal characteristics. *Anopheles splendidus*,  $N$  = 4, Ban Mae Hoi as above;  $N$  = 3, Mae Hong Son as above. *Anopheles jamesii*,  $N$  = 6, Ban Takian Duan as above. *Anopheles ramsayi*,  $N$  = 1 Ban Mae Hoi as above.

Pinned reference specimens from which ovaries were cropped are housed as follows: Taiwanese specimens are held by Mr. P. S. Chen, Taiwan Provincial Institute of Infectious diseases, 161 Kun Yang Street, Nankang, Taipei; Philippine material is held by Mrs. Adela Cagampang-Ramos, USAF Occupational and Environmental Health Lab (AFSC), APO San Francisco, U.S.A. 96274; Thai material is partly held by one author (B. A. Harrison). Some reference material consists of family material where larval and pupal skins are associated with the pinned adults (also held by B. A. Harrison).

Field, cytological, and photomicrographic methods are documented (Green and Hunt (1980) and references therein). The methods used in handling data and working out homologies between banding sequences along the polytene chromosomes have been documented, together with the method (Green 1982b) underlying the presentation of data in the

photomaps in Figs. 1–4. A brief account is given here of the latter point.

A standard arrangement was chosen arbitrarily as *superpictus* for the series Neocellia (Coluzzi *et al.* 1970). We have never worked with this species, but have worked with *stephensi* which Coluzzi *et al.* (1970) compared with *superpictus*. Thus we have been able to refer back to standard through *stephensi*. We have referred to the standard arrangement in all figures using the chromosomes from various species other than *superpictus*. The simplest example is Fig. 3. Both *maculatus* and *ramsayi* are homosequential (= same banding pattern) with *superpictus* in arm 3. Thus the block designations, A, B, C, D, etc., read in alphabetical sequence indicating the standard arrangement, but using arm 3 from *maculatus*. These blocks are segments of the chromosomes that have remained intact during the evolution of the group since the origin of the common, single ancestor. In Fig. 4 the figured arm 5 comes from *philippinensis* which is homosequential with *superpictus*. Here there are no block designations referable to Neocellia because there is no other arm 5 shown in the figure. The block designations shown on arm 5 refer to homologous blocks in the series *Myzomyia* and shown in Green (1982b). Where block designations have a dot over them in all the figures, those blocks are inverted with respect to standard arrangement, i.e., A is the mirror image of  $\dot{A}$  in terms of banding pattern. Thus we show an arm 4 from *stephensi* to represent standard and show  $\dot{A}$  to indicate the one inversion difference, 4y, between these two species, and the rest of the blocks are in alphabetical sequence, i.e., the same as standard. There is one exception to this use of block designations (apart from those on arm 5, Fig. 4, mentioned above). In an effort to simplify visualizing homologies of *annularis* it is referred to *philippinensis* and so the block designations between these two in Fig. 3 are not directly referable to standard.

To demonstrate the reference to the standard arrangement, arm 2a<sub>el</sub> in *philippinensis* is used as an example. The standard arrangement of arm 2, i.e., that found in *superpictus*, is achieved if the *philippinensis* arm 2 is inverted for 2a<sub>el</sub>. It does not matter in which order these inversions are made since no single breakpoint of one of these inversions falls between breakpoints of another inversion, i.e., none overlaps another even though 2e is included within 2l. Where inversions do overlap then one is obliged to start the sequence to standard from the right-hand end of the inversion formula of a particular species.

### Results and discussion

Anophelines of the subgenus *Cellia* have two pairs of autosomes and one pair of sex chromosomes. There is evidence that whole arm translocations have occurred which are judged from differing arm associations in different groups of species (Coluzzi *et al.* 1970; Green and Hunt 1980). All the species dealt with here showed the same arm association that has been reported for other members of Neocellia, i.e., one autosome consists of arms 2 and 5, the other, arms 3 and 4 (see Green and Hunt (1980) for this designation of arms). Chromo-

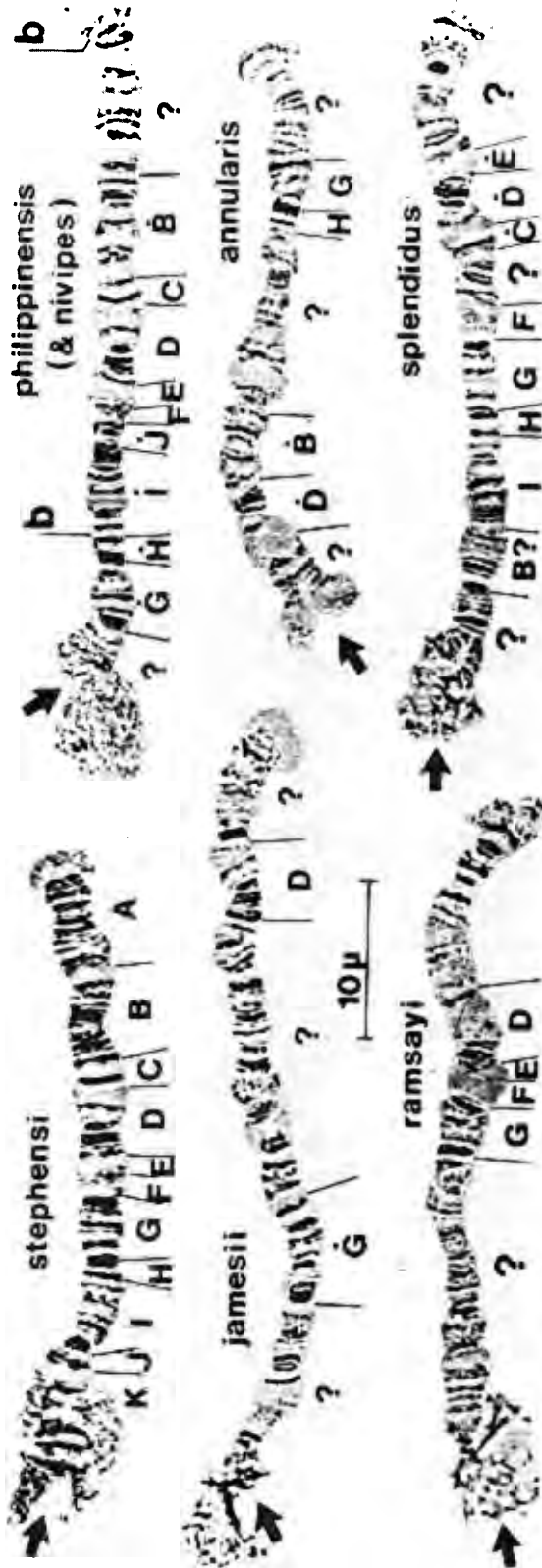


FIG. 1. X chromosomes from the ovarian polytene sets of the *Anopheles annularis* species group. Arrows here and on other figures indicate the centromeric ends of the arms. Block homologies refer to arbitrary standard arrangement found in *superpictus* (homosequential with *stephensi*). In this and all the other plates when a block designation has a dot over it, then it is reversed with respect to the standard; i.e., A is the mirror image of A. "??" indicate uncertain or unknown homologies.

somal rearrangement in this group appears to be solely due to fixation of alternatives of paracentric inversions.

#### *Inversion alternatives fixed within species*

Homologies between the various X chromosomes are incomplete so that there are no data available for these chromosomes in terms of postulated inversion sequences. They are presented in Fig. 1 for reference purposes and to show the limited homologies found between them. Coluzzi *et al.* (1970) reported that *stephensi* is homosequential with standard for the X chromosome.

Figure 2 shows the various arm 2 arrangements. The hypothetical *Myzomyia* arm 2hijklm (Fig. 2) is produced from material from *culicifacies* and the inversions bear no relationship to similarly designated inversions within the *Neocellia* data set. *Myzomyia* data in Figs. 2 and 4 are from Green (1982b) with new, additional information of interseries homologies between *Myzomyia* and *Neocellia* for arm 2. The block designations indicated on the *Myzomyia* sequence show the rearrangement between this hypothetical arm and *superpictus*, and are the same block designations used throughout Fig. 2.

Rearrangement of arm 2 has been extensive in both *annularis* and *splendidus*, as can be judged from the block rearrangements in these arms when compared with that of *philippinensis*. These rearrangements are unique to each taxon and involve several cases of common breakpoints for different inversions. A minimum of five may account for the *splendidus* rearrangements and a minimum of nine for *annularis*. However, several different inversions can be postulated, each giving sequences with the minimum number of five or nine, respectively. Which of these actually occurred in history cannot be determined. Obviously, there is no phylogenetic information in derived states unique to single taxa and so none of the several possible inversion sequences for either of these two taxa has been presented. The raw data are the relationships of the segments of chromosomes that have remained intact during the evolution of the group and these are given in Fig. 2. The lengthy sequences of postulated inversions for arms 3 and 4 in *annularis* had been produced before the logic outlined above had occurred to us. They should be treated with extreme caution should new information reveal that other taxa share some of the block relationships presently unique to *annularis*.

We have revealed an error in Green's (1982a) homologies for *pulcherrimus*. This taxon proved to be homosequential with *philippinensis* for inversions 2l (Fig. 2) and 3akl (Fig. 3). Accordingly 2l in the earlier

work should be replaced by 2l shown here in Fig. 2. The error with arm 3 is more complex and arose because the *pulcherrimus* rearrangement was very different from all others (Green 1982a). Inspection of the same *pulcherrimus* data along side close relatives in the *annularis* group (see Fig. 5) revealed these errors which involved small segments of the arm. This finding emphasizes the need for comparative work between several close relatives at the same time and not the consideration of isolated species against material from relatively distant species. Inversions 3ghi in Green (1982a) should be totally disregarded. Thus our 3i is quite different from the former 3i, and 3gh refer currently to no inversions within the series.

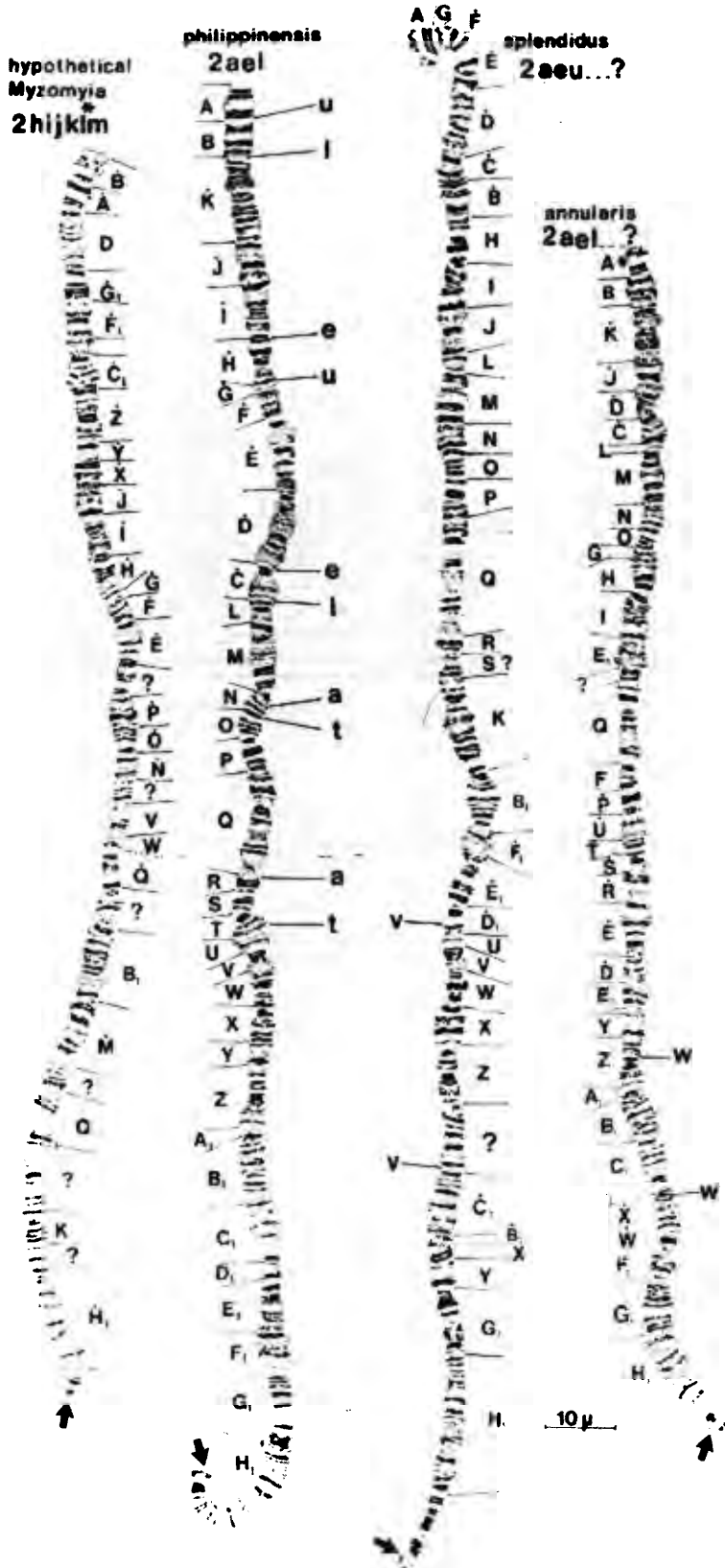
We have taken the data from Green (1982a), Green *et al.* (1985), and Green and Baimai (1984), together with the data reported here to give an informational display (Fig. 5) of the inversion formulae of all cytologically known species in the series *Neocellia*. The idea for such a display was in Carson's (e.g., Carson 1983) work on the picture-winged *Drosophila* of Hawaii and is explained in the legend of Fig. 5 which is virtually word for word from Carson's Fig. 1 (1983).

#### *Cladistic analysis*

Green (1982b) gave a detailed argument for the application of cladistic principles in trying to determine phylogenetic relationships from polytene chromosome data because most *Drosophila* workers feel that no time direction can be determined from such data. He (Green 1982b) was following the general argument of Farris (1978) that chromosome data are no different from other data which are generally used in cladistic analyses. Cladistic analysis has already been employed by cytogeneticists using Giemsa-banding patterns in mitotic chromosomes, e.g., in peromyscine rodents (Yates *et al.* 1979).

Basically, for each inversion one wishes to know which alternative is ancestral (plesiomorphic) and which is derived (apomorphic). Clearly relationships within the group under study can be determined only from the sharing of apomorphic states, i.e., synapomorphies, those transformations that have occurred during the evolution of the group and that are shared by two or more members of the group. Plesiomorphic characters predate the origin of the group have nothing to tell us about relationships within the group. Two criteria are available for suggesting which inversion alternative is ancestral and which is derived. First, the alternative which is common within the group is probably ancestral. Secondly, that alternative which is shared with a group of species outside the one under study is

FIG. 2. Arm 2 from ovarian polytene chromosomes of the *Anopheles annularis* species group. The *Myzomyia* arm 2hijklm is the hypothetical ancestral arrangement from that series (Green 1982b), and these inversions bear no relationship to similarly designated inversions on arm 2 in *Neocellia*. *Anopheles nivipes* is homosequential with *philippinensis* except it is fixed for 2l.





probably ancestral. These are not independent criteria since both simply depend on relative commonness of one alternative over the other either within the group under study, or within this group and groups closely related to it. When these two criteria give contradictory answers, e.g., an alternative is rare within the group but also occurs as the only alternative in the out-group, then the latter criterion is considered the more important indication, e.g., 3a is common in *Neocellia*; only *stephensi* has 3+<sup>a</sup>, but this alternative occurs in *Myzomyia*, the out-group. Hence 3+<sup>a</sup> is probably the ancestral sequence.

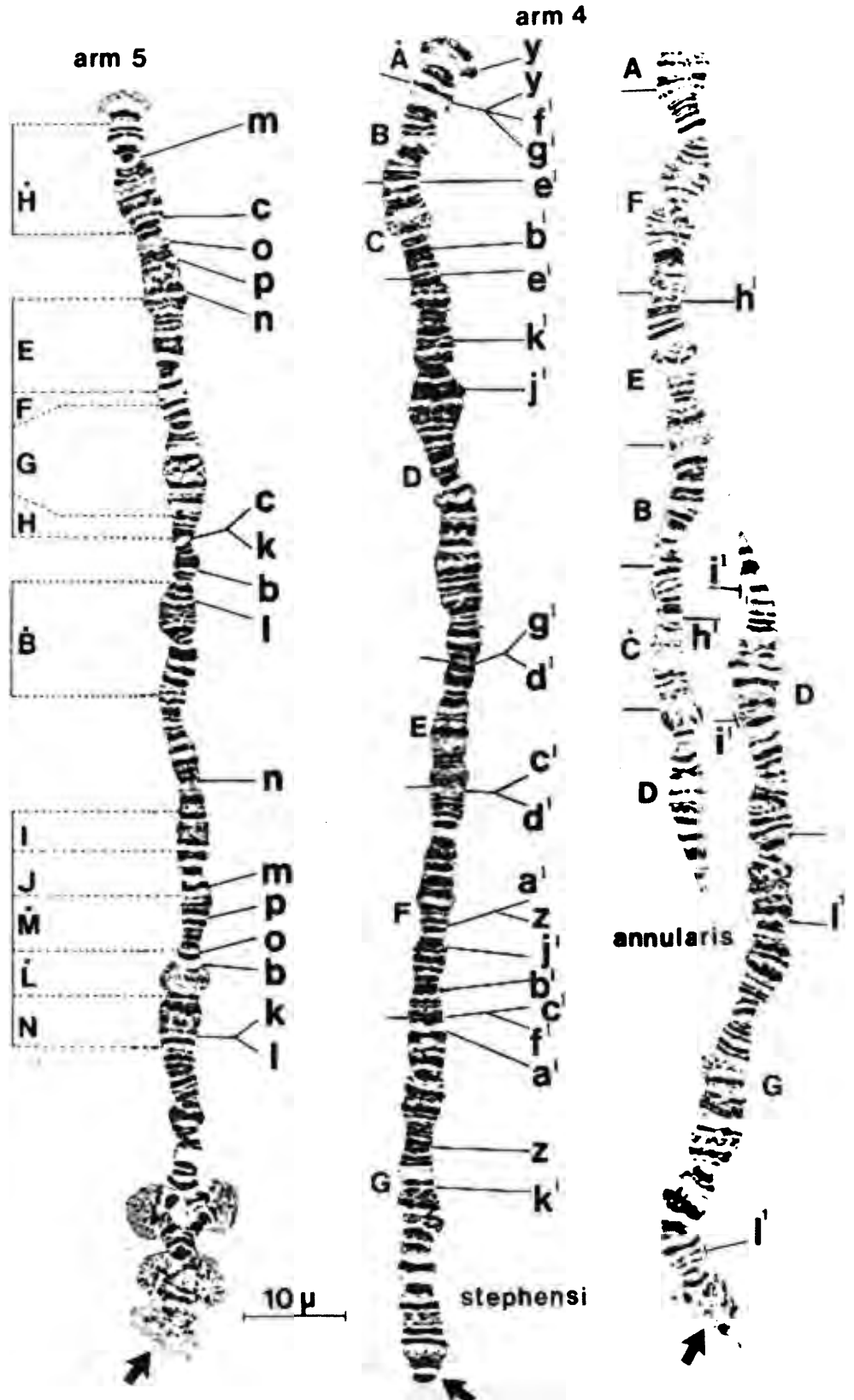
We have used *Myzomyia* for out-group comparison and Green (1982b) used *Neocellia* as the out-group for the analysis of *Myzomyia*. Slowly intergroup homologies are accumulating and in the case of arm 4, total homologies are known between the two series. Unfortunately little is known about other species groups in terms of interspecific homologies so there is, as yet, no other out-group to which we might resort in determining which of the postulated inversion alternatives for the arm 4 inversions, i.e., p, q, and r, are ancestral or derived in the common ancestor to the series *Myzomyia* and *Neocellia*. Despite this encroaching limitation, we repeat the suggestion of Green (1982b) that cladistic analysis is highly heuristic in that it leads to small, explicit hypotheses, i.e., which inversion alternative is derived (shown in Fig. 6 by black squares), which can be easily tested, and possibly rejected, in the light of further information. For example, Green (1982b) decided that the alternative for 2m, fixed in *leesoni* Evans, *fluviatilis* James, *culicifacies* Giles, and *demeilloni* Evans, was derived within *Myzomyia*, although he questioned this in the light of the fact that *demeilloni* showed impossible relationships within the group. Our work shows that he was wrong, as judged by out-group comparison, and the alternative seen in the taxa mentioned above was in fact the ancestral alternative (within *Myzomyia*). This is because a small, terminal inversion (as yet not designated) involving blocks A and B in Fig. 2 produces the arrangement seen in all other series within *Cellia* (C. A. Green, unpublished data). This inversion overlaps the alternative of 2m seen in the *leesoni* clade and probably occurred before 2m in the early evolution of the species within *Myzomyia*.

Interseries homologies presented here are far more than those demonstrated earlier (Green 1982b), particularly in the case of arm 2 and arm 5 (Figs. 2 and 4,

respectively). A simple example of the use of these interseries homologies for the purpose of out-group comparison is that afforded by 2e. Is the alternative seen in *philippinensis* the ancestral or derived sequence? We need to find the relationship of blocks C-D and H-I in the *culicifacies* arm 2 since these two pairs of blocks bracket the two breakpoints of 2e. Of these, only H/I is evident and is the same as that seen in *philippinensis*. Therefore the alternative in the latter is probably the ancestral because it is shared with the out-group. It does not matter that we cannot find blocks C/D in the *Myzomyia* arm 2, since the one breakpoint necessarily infers the other. Thus for inversion 2e, *philippinensis* shows a white square in Fig. 6. In Fig. 4 the arm 5 shown comes from *philippinensis*, and the blocks indicated by broken lines are the same blocks shown in Green (1982b) for arm 5 in *Myzomyia*, i.e., represent interseries homologies for this arm. The block designations near the arm 5 map come from Green (1982a). Only 5n has both breakpoints in segments of unknown interseries homology, though breakpoints of 5c are close to boundaries between unknown and known homologies of chromosome segments and so it too might be unavailable for out-group comparison. Thus, *philippinensis* is fixed for the ancestral sequences of all the other arm 5 inversions indicated in Fig. 4. Hypotheses involving arm 3 and arm 4 (Figs. 3 and 4, respectively) were made from Figs. 2 and 3 in Green (1982b).

The level of rearrangement within *Neocellia* is such that limited phylogenetic information is available as seen in Fig. 6. There are no lengthy sequences of cladistic events to be found in the data. Only three groups can be seen which involve 12 of the 15 species. Neither within nor between these groups is there any indication of relationships except for membership to the *maculatus* complex. The Afrotropical species form one group, which is reinforced by the extrinsic data from their geographic distribution; *pulcherrimus*, *annularis*, *philippinensis*, and *nivipes* form a second group; and *superpictus*, *stephensi*, and the *maculatus* complex represents a third group. Notice that these groupings are not equally secure. The *stephensi* group members share only one fixed derived alternative, i.e., 2e. Notice that the data for 3a are in conflict with those for 2e. Inversion 3a suggests that *stephensi* and the common ancestor to the other species arose from the first speciation event in the group where *stephensi* retained the ancestral alternative for 3a, i.e., the derived alternative arose in the clade leading to all other species in the group.

FIG. 3. Arm 3 from ovarian polytene chromosomes of the *Anopheles annularis* species group. Species taxa names in parentheses indicate they are homosequential with the figured sequences. *Anopheles jamesii* differs from *maculatus* by inversion 3q. Blocks are homologous between *maculatus*-*splendidus* and between *annularis*-*philippinensis*, but not between these two pairs. Invert 3kl in *philippinensis* to derive the *superpictus* (= *maculatus*) arrangement. Notice 3kl breakpoints are close together and somewhat confused by the regular puff immediately distal to the outer 3k breakpoint (from the centromere). These facts account for the errors in interpretation of *pulcherrimus* by Green (1982a).



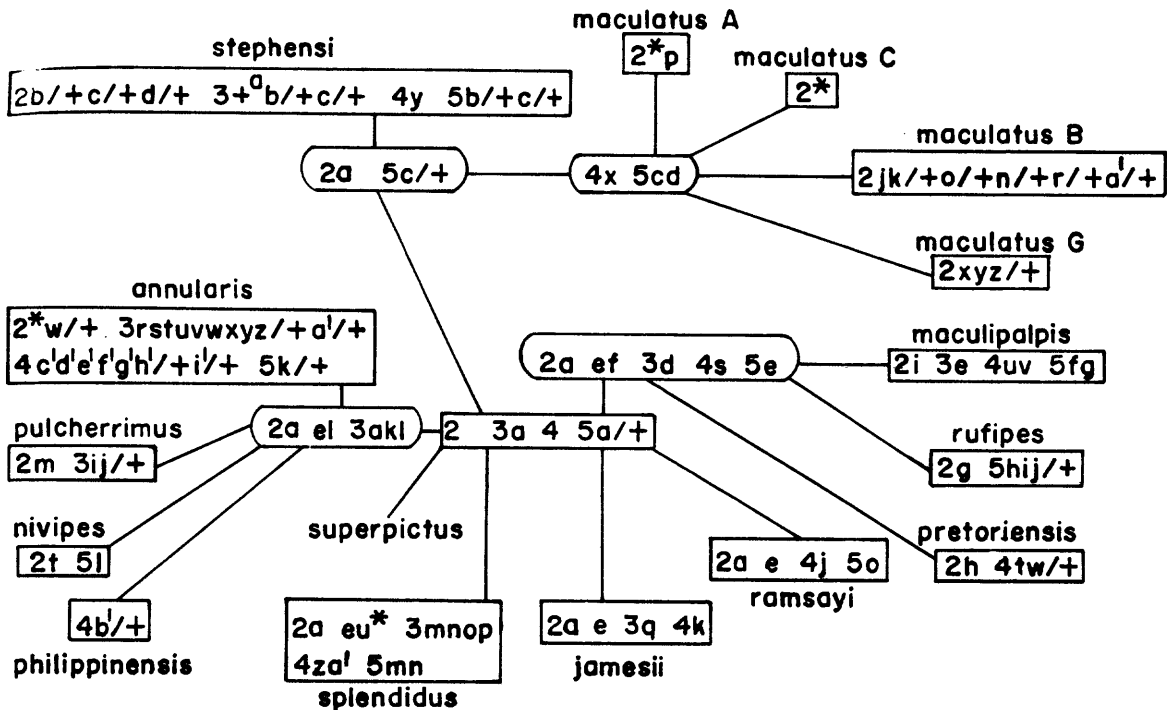


FIG. 5. Informational display of polytene sequence formulae in 15 species of the series *Neocellia*, *Anopheles* (*Cellia*). Each inversion is represented by a lowercase letter (employed in the order of discovery) used as a suffix to the number of the chromosome arm. The large number of inversions in some arms have made it necessary to use the alphabet more than once, i.e., inversions of arm 4, discovered after 4z, are designated 4a<sup>i</sup>, 4b<sup>j</sup>, etc. (These symbols are read "four b one" etc.) When symbols stand alone, e.g., 2e, this means that the sequence is present in the homozygous state, i.e., fixed, within the particular species. Where both sequences occur in the polymorphic state within a species, they are represented thus 5c/+ where "+" refers to the standard sequence. In some cases, repetition in the display of sequences shared by two or more species has been avoided by adding to the diagram inferences that such species shared a common single intermediate population having a certain inversion composition. The inversion formulae of these inferred intermediate populations are given within boxes with rounded ends. The conditions found within existing species are placed within rectangular boxes. This explanation is almost verbatim from Carson (1983, Fig. 1). \* indicates inversion sequences which have not been designated as explained for *annularis* and *splendidus* in the text. The number of inversions on arm 2 in *maculatus* species A is four, and in species C three.

The most likely interpretation of this single conflict of data in Fig. 6 is that 3a was polymorphic through several speciation events and fixation of 3a in *superpictus*–*maculatus*, and the other species is an example of parallelism. Parallelism is probably common with inversion fixation (Farris 1978; Green 1982b) and so evidence of relationship based on a single inversion, i.e., 2e, is highly suspect. The sharing of the derived alternative 5c, fixed in the *maculatus* complex and polymorphic in *stephensi*, might suggest a recent common ancestor between these taxa, but polymorphic inversions can persist through several speciation events as pointed out above. There are no indications of any relationship of *splendidus*, *jamesii*, and *ramsayi* save for their inclusion within *Neocellia* indicated by 3a (see Green 1982b) for discussion of the derived status of 3a

within *Neocellia*).

Originally the report did not include Fig. 5. This was added on the advice of a referee for the journal. It follows, almost exactly, the scheme used by Carson and his colleagues (e.g., Carson 1983) to show interrelationships between species of Hawaiian *Drosophila*. The construction of the figure showed that cladistic ideas of relationship have impinged upon the use of an arbitrary standard as seen by the change of standard to show inversions within it. Carson's scheme obliges one to have standard bare of any inversion formula and so gives a "specialness" to standard that is arbitrary. For example in his Fig. 1 (Carson 1983), 4b holds a central position in the figure. Cladistic principles alone would suggest that it is the ancestral alternative and that 4+<sup>b</sup> is probably derived within the *Drosophila grimshawi*

FIG. 4. Arm 5 and 4 from ovarian polytene chromosomes of the *Anopheles annularis* species group. The arm 5 is from *philippinensis* and is homosequential with *superpictus*. Other species differ from *philippinensis* arm 5 or the *stephensi* arm 4 as indicated in Fig. 5. Arm 4 *stephensi* differs from *superpictus* by inversion 4y.

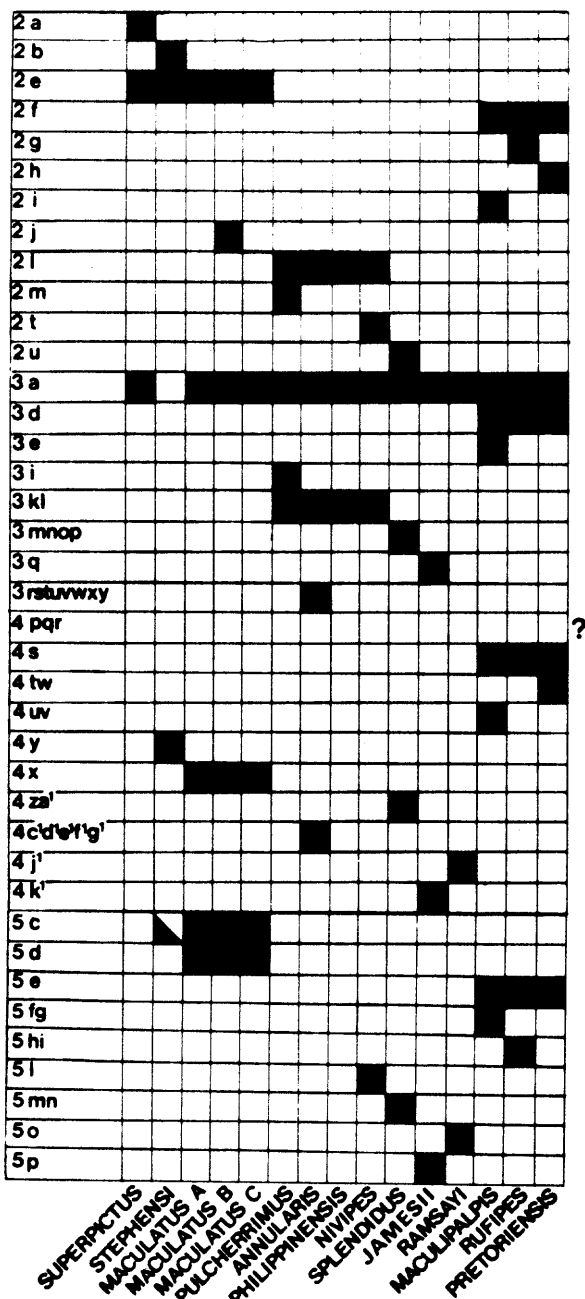


FIG. 6. Apomorphies for alternatives of autosomal inversions in some species taxa in the series Neocellia of *Anopheles* (*Cellia*). Note that nine inversions and five on the arm 2 of *annularis* and *splendidus*, respectively, are not shown (see text). Black squares indicate that the derived alternative is fixed in the indicated species; white squares indicate the ancestral sequence except for 4pqr whose status are presently unknown.

group, because the latter is a much smaller group of species than those of the rest of the data set. The perceptive reader will notice an inconsistent impact of cladistics in Fig. 5. Inversion 2e is probably derived in

the *superpictus stephensi maculatus* group and so an ancestral intermediate for all the other species should be shown having 2+<sup>c</sup>. Eventually far greater problems in inversion nomenclature will occur when the Neocellia and Myzomyia series are linked by total chromosome homologies into one group, for then there will be two arbitrary standards, e.g., *superpictus* and *funestus*. The minor inconsistencies within this work should await the major revision needed when Myzomyia and Neocellia are joined together by total homologies.

Polymorphic inversions found within species are indicated in Fig. 5, and are shown on the chromosomes of respective taxa in Figs. 2-4 and the plates in Green (1982a). Data from the *maculatus* complex are documented in Green *et al.* (1985) and Green and Baimai (1984).

#### The philippinensis nivipes complex

This work has some practical implication where interest exists for malaria parasite transmission by so-called *philippinensis*. Adults of both *philippinensis* and *nivipes* are very similar morphologically and the species are distinguished on pupal characters. It is not possible to be certain of identification made solely from the adults. The chromosomes remove this ambiguity at least for Thailand since the two species differ by 2t and 5l. Thus adults can now be identified directly without recourse to checking laboratory-reared offspring from wild-caught females for the diagnostic pupal morphology.

There is an indication from the data that *nivipes* might consist of two species. Samples from the west and north of Thailand show fixation of inversion sequence X+<sup>b</sup>, while those from the southeast are fixed for Xb. A definitive answer to the possibility will come from seeing how these chromosomes segregate in an area where they occur together in nature.

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