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Genetics of esterases and 6-phosphogluconate dehydrogenase in the *Anopheles maculatus* complex

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Summary

Electromorphic variation for some esterases and 6-phosphogluconate dehydrogenase enzymes in the *Anopheles maculatus* complex is controlled by four loci which are unlinked to sex. Esterase loci are linked to each other: *Est-1* – 36% – *Est-4* – 16.5% – *Est-3*; but unlinked to *Pgd-2*. Linkage data were obtained by selfing the F_1 generation from selected parents and analysing genotypes in the F_2; the classical dihybrid-cross. The analysis consists of testing observed data for goodness of fit to a) ratios expected from Mendelian ratios without linkage and b) if they do not fit then computing a likely degree of linkage and computing expected ratios with such linkage for further tests. Confidence limits are given for the most likely levels of linkage. This method can provide useful information for population-genetic studies on anopheline mosquitoes, whose laboratory rearing is generally difficult. Through indirect evidence, the enzyme loci are correlated to polytene chromosomes. The esterases probably lie on chromosome three (polytene arms 3 and 4) and *Pgd–2* on the second chromosome (arms 2 and 5).

Key words: esterases; 6-phosphogluconate dehydrogenase; *Anopheles maculatus* complex; electromorphic variation; linkage group/polytene chromosome correlation.

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Introduction

Anopheles maculatus is a highly variable taxon with respect to morphology and the ability to transmit malaria (Reid, 1968). This suggests that it may include two or more biological species. Four species are evident from analysis of polytene chromosome arrangements in wild-caught *A. maculatus* from Thailand (Green and Baimai, 1984; Green et al, 1985a). We are interested in developing a more practical means of identifying species in the *A. maculatus* complex during routine malariometric studies than that provided by the chromosomal rearrangements. Electromorphic variation could provide such a method as has been used with the freshwater species of the *A. gambiae* group (Mahon et al., 1976; Miles, 1978). Indeed, since Ayala and Powell (1972) introduced the use of 'diagnostic' electromorphs to distinguish morphologically-cryptic species in *Drosophila,* numerous insect species have been analysed electrophoretically to provide information about their systematics (Berlocher, 1984). Examples in which the technique has lead to the discovery or elucidation of morphologically-cryptic species, are the *Simulium jenningsi* group of black flies (May et al., 1977), the *Perthida glyphopa* complex of Jarrah leaf miners (Mahon et al., 1982) and the *Tabanus nigrovittatus* group of salt-marsh horse flies (Sofield et al. 1984).

Apart from a means of identification in the *Anopheles maculatus* group, electromorphic variation can provide intrinsically interesting, population-genetic data. In such studies one needs to know genetic linkage relationships of the loci coding for proteins among themselves, and with the many polymorphic inversions on chromosome arm 2 and the X chromosome that occur in Thai populations of *A. maculatus* (Green and Baimai, 1984; Green et al., 1985a).

Esterase loci in *A. culicifacies* (Dubash et al., 1982) and in *A. stephensi* (Iqbal et al., 1973; Sakai et al., 1983) have been correlated to polytene chromosomes. These workers used classical formal genetic methods in their studies. Such breeding schemes are technically difficult with many anopheline mosquitoes due to labour intensive needs in laboratory breeding and partial refractoriness to laboratory colonization in the case of other species. The work in this report used a simplier breeding scheme and gives the formal genetics and a probabilistic estimation of linkage relationships of some genes controlling esterases (EST, E.C. 3.1.1.1.) and 6-phosphogluconate dehydrogenase (PGD, E.C. 1.1.1.44.) variation in *A. maculatus.* Furthermore the probable correlation of these genes to polytene chromosome elements is possible since Green (1982b) has given details of homology between these chromosomes in *A. stephensi* and *A. maculatus* and whole-arm homologies between the *A. stephensi* group and *A. culicifacies* (Green, 1982a).

Materials and Methods

Two laboratory colonies were used (housed at AFRIMS Lab., Bangkok, Thailand). One originating from Malaysia, called Kuala Lumpur (KL), is a subcolony of one housed in the Institute for Medical Research, Kuala Lumpur, Malaysia. The other (HK) was established from about 200 eggs from females caught biting man at Ban Huai Kuum, Chonburi Province, Thailand in 1979. Both colonies are maintained by hand mating, since neither will mate naturally in the insectary. HK belongs to species B, and KL to form E of *A. maculatus* complex and which are probably conspecific (Green and Baimai, 1984; Green et al., 1985a).

Single pair matings were made; the male was immediately stored at –70° C as was the female once she had laid a clutch of eggs. Parents and some progeny were run individually on the same gel during electrophoresis. The F_1 of selected families were selfed to provide F_2. Table 1, columns 8, 9 and 10 together with the coding in rows 3 to 8 provide the genotypes of the parents from which F_2's were produced.

Electrophoresis involved the homogenizing of individual insects and absorption onto two 2×6 mm wicks for insertion into different gel systems (Steiner and Joslyn, 1979) and the following procedures: (i) *Esterases.* Horizontal polyacrylamide gels with TEB buffer (Green, 1977) were used. Runs were terminated after about 2 h or when the bromophenol blue marker had migrated anodally 11.5 cm (under these same conditions, a normal human hemoglobin marker migrates 3.5 cm). The staining (Steiner and Joslyn, 1979) included both alpha- and beta-naphthyl acetates as substrates. *Est-3* was scored after 20 min incubation at 37° C, *Est-4* after a further 25 min, and *Est-1* after a total of 2 h incubation. (ii) *Dehydrogenases.* A 7% polyacrylamide gel system (Tan and Teng, 1979) was used based on a TEMM buffer system (Spencer et al., 1964). Samples per gel were doubled by making two stacks of inserts; one 6 cm anodal to the other. All gels included two slots for reference markers made from pools of three to four KL insects to ensure accurate scoring. The run was terminated after about 2 h or when the bromophenol blue marker had migrated anodally 8 cm. Staining (Steiner and Joslyn, 1979) was modified for PGD by using Tris-HCl at pH 8.0 and adding PMS at the start. This enzyme system was scored after a 30 min incubation at 37° C. After removal of the PGD stain, the same gel was stained sequentially first for glucose phosphate isomerase (GPI, E.C. 5.3.1.9.) and then for phosphoglucomutase (PGM, E.C. 2.7.5.1.). The last two enzymes showed no variation in our material so we discontinued this sequential staining.

The following enzyme systems were screened using 7% polyacrylamide gels and various buffer systems (Steiner and Joslyn, 1979) but showed no variation. Xanthine dehydrogenase (XDH, E.C. 1.2.1.37.), 2-octonol dehydrogenase (ODH, E.C. 1.1.1.73.), alpha-glycerophosphate dehydrogenase (α-GPDH, E.C. 1.1.1.8.), malic dehydrogenase (MDH, E.C. 1.1.1.37.), malic enzyme (ME, E.C. 1.1.1.40.), hexokinase (HK, E.C. 2.7.1.1.), aldehyde oxidase (ALDOX, E.C. 1.2.3.1.) and beta-hydroxybutyrate dehydrogenase (β-HBDH, E.C. 1.1.1.30.).

Results

There were seven regions of esterase activity, of which three showed probable genetic variation (Fig. 1A). These were called *Est-1* with the fastest relative mobility, *Est-3* of intermediate mobility, and *Est-4* with the slowest mobility. The esterase electromorph designations roughly equate to 1 mm per unit from the "100" morph, e.g. "107" is about 7 mm anodal to "100" and "95" is 5 mm cathodal to "100". *Est-4* showed a pink coloration, indicating use of the beta-naphthyl acetate as a substrate (the slowest zone seen on Fig. 1A was also pink, but variation appeared non-genetic). All other zones showed the typical black/brown color of alpha-naphthyl acetate utilization. Two zones of PGD activity were seen, an anodal invariate zone called PGD 1 and the variable zone

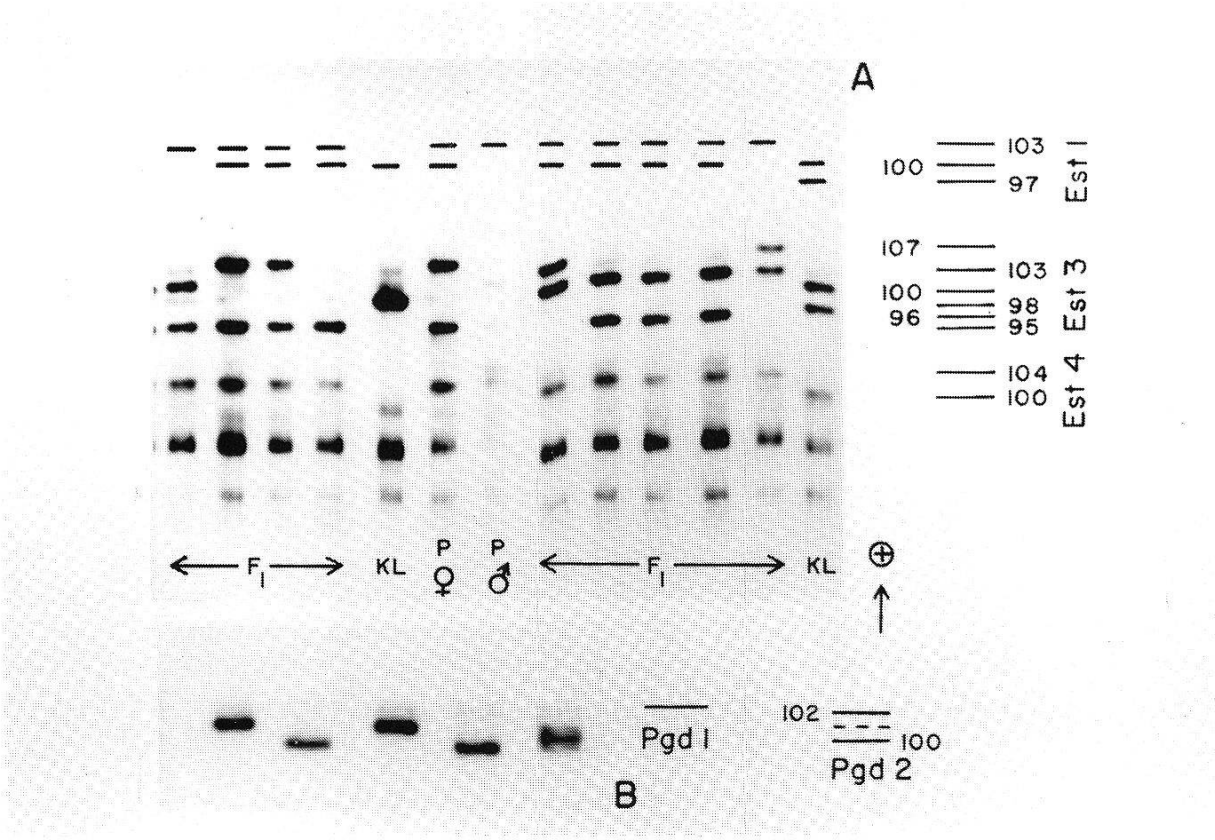

Fig. 1. *A.* Esterase phenotypes of individuals from a single family of *Anopheles maculatus*. The genetic interpretation is drawn on the right of the figure. In this black/white plate phenotypes for *Est-4* are confused (in color there is no confusion due to its pink coloration): the two KL markers are homozygous for $Est\text{-}4^{100}$ and the rest for $Est\text{-}4^{104}$. Parental phenotypes were: female: $Est\text{-}1^{103/100}$, $Est\text{-}3^{95/107}$, $Est\text{-}4^{104/104}$; male: $Est\text{-}1^{103/103}$, $Est\text{-}3^{103/95}$, $Est\text{-}4^{104/104}$. The *Est-1* phenotypes have been inked-in because they were weak-staining. In the zymogram $Est\text{-}3^{98}$ is absent. *B.* 6-phosphogluconate dehydrogenase phenotypes of individual insects; the genetic interpretation is drawn on the right. The dotted line represents the hybrid band.

PGD-2 (Fig. 1B). The various alternatives at each of the presumptive loci and seen in both colonies are diagramed to the right of the gel in Fig. 1A and B.

Given the above hypotheses as to the likely genetic control of the observed genotypes, various crosses were made for variation at each locus. The observed phenotypic frequencies of the progeny were tested against expected frequencies deduced from Mendelian ratios. In no case did the observed frequencies show a significant departure from the expected frequencies. In the following summary of analyses of these crosses for each locus, the first sample of 202 individuals (201 in crosses involving *Est-4*) refers to the raw data in Table 1. The other tests listed below show that esterase electromorphs which were not present in the crosses used to generate the data in Table 1 are also inherited in a Mendelian fashion. The raw data are not presented here for the sake of brevity but are available on request from CAG.

Table 1. Observed frequencies of electromorph genotypes of F_2 individuals of *Anopheles maculatus* used to estimate linkage relationships between three esterase loci and one 6-phosphogluconate dehydrogenase locus. Each of the 202 F_2 were scored for all four loci and Rows 3–8 show the genotypic frequencies for all pair-wise combinations of the loci. Row 1 shows the Mendelian ratios expected in the absence of linkage and Row 2 shows the expected frequencies based on these ratios and the sample size of 202 F_2 scored. These expected frequencies were used to test 'goodness of fit' of observed frequencies assuming a Null hypothesis (see text for analysis and foot-note for explanation of coding for, a, A, b, and B in rows 3–8).

	F_2 Genotypes									
	1 aB/aB	2 Ab/Ab	3 AB/Ab	4 Ab/ab	5 AB/aB	6 aB/ab	7 Ab/aB	8 AB/ab	9 ab/ab	10 AB/AB
1:	1	1	2	2	2	2	2	2	1	1
2:	12.6	12.6	25.2	25.2	25.2	25.2	[50.4]		12.6	12.6
3:	1	3	12	10	18	11	[85]		29	32
4:	6	8	26	17	27	17	[65]		17	18
5:	12	12	28	20	23	26	[54]		10	16
6:	6	13	21	22	37	22	[55]		13	13
7:	14	17	21	22	28	17	[60]		9	14
8:	6	9	31	18	29	20	[63]		14	12

Note that the genotype codes indicate the parental genotypes as AB/AB (KL colony), and ab/ab (HK colony). Columns 1–7 show recombinant genotypes and columns 8–10 show the unrecombined genotypes recovered in the F_2.

Code for rows 3–8:

3. Observed genotypic frequencies in progeny of F_1 for comparison of *Est-4* v. *Est-3*; B = 4^{100} and A = 3^{96} from the KL colony, and b = 4^{104} and a = 3^{95} from the HK colony.
4. *Est-4* v. *Est-1*; where B = 4^{100}, b = 4^{104}, A = 1^{97} and a = 1^{103}.
5. *Est-4* v. *Pgd-2*; where B = 4^{100}, b = 4^{104}, A = 2^{100} and a = 2^{102}.
6. *Est-3* v. *Pgd-2*; where A = 3^{96}, a = 3^{95}, B = 2^{100} and b = 2^{102}.
7. *Est-1* v. *Pgd-2*; where A = 1^{97}, a = 1^{103}, B = 2^{100} and b = 2^{102}.
8. *Est-1* v. *Pgd-3*; where A = 1^{97}, a = 1^{103}, B = 3^{96} and b = 3^{95}.

Parental phenotypes were: KL male *Est-1*^{97}, *Est-3*^{96}, *Est-4*^{100}, *Pgd-2*^{100} and HK female *Est-1*^{103}, *Est-3*^{95}, *Est-4*^{104}, *Pgd-2*^{102}. The F_2 individuals came from five pair-matings of the F_1.

Esterase 1

1. 97/103×97/103, N = 202, $\chi^2_{(2df)}$ = 3.03. 2. 97/100×97/100, N = 112, $\chi^2_{(2df)}$ = 5.21. 3. 103/103×100/103, N = 85, $\chi^2_{(1df)}$ = 1.42.

Esterase 3

1. 95/96×95/96, N = 202, $\chi^2_{(2df)}$ = 3.52. 2. 96/100×96/100, N = 82, $\chi^2_{(2df)}$ = 0.22. 3. 95/103×95/103, N = 55, $\chi^2_{(2df)}$ = 3.25. 4. 95/95×95/107, N = 50, $\chi^2_{(1df)}$ = 0.08. 5. 95/98×95/107, N = 30, $\chi^2_{(3df)}$ = 4.06.

Esterase 4

1. 100/104×100/104, N = 201, $\chi^2_{(2df)}$ = 1.93.

6-phosphogluconate dehydrogenase 2

1. 102/100×102/100, N = 202, $\chi^2_{(2df)}$ = 0.81.

Electromorphs in each zone are controlled by single loci per zone with codominant alleles. Since heterozygous males were observed for all loci, none of the loci lie on the sex chromosomes; anophelines have a XY (males)/XX (females) chromosome system. We tested for possible linkage to an autosomal sex mechanism such as that found in culicines, though unknown in anophelines. The same 202 F_2 individuals in Table 1 showed no significant association with sex (*Pgd-2*, $\chi^2 = 4.44$, $P>0.10$; *Est-1*, $\chi^2 = 0.76$, $P>0.50$; *Est-3*, $\chi^2 = 0.20$, $P>0.9$; *Est-4*, $\chi^2 = 2.15$, $P>0.30$; df = 2 in all cases).

The esterases (Fig. 1A) are evidently monomers as heterozygotes show only two bands. Since *Pgd-2* (Fig. 1B) shows the characteristic three bands in heterozygous individuals, it is probably a dimer. We noticed a very distinct artifactual band in Pgd from insects that died sometime prior to being frozen (e.g. females after egg-laying). The artifact occurred at about 2.5 cm from the origin; *Pgd-2* migrated between 0.8 and 1.0 cm and *Pgd-1* moved 1.2 cm.

The data in Table 1 are analyzed to obtain the probability of recombination, q, and its confidence interval, CI, for each pair of loci. Our method of analysis is to: a) obtain a "best guess" estimate of q based on phenotypes of the observed data using Steven's (1939) product ratio; b) determine the expected genotype frequencies using this q value; c) compare the expected frequency distribution with the observed frequencies over the genotype classes using the G-test (Sokal and Rohlf, 1981) statistic (with one less degree of freedom since the "best guess" q estimate is derived intrinsically from the observed data). If the two distributions are significantly different, reject the hypothesis that the mechanism of genetic inheritance involves only Mendelian ratios and linkage phenomena (since one cannot expect that the "best guess" q derived from *phenotype* data will best fit the *genotype* model, it would be wise to evaluate G test values for nearby q's even if the "best guess" q is rejected). If the distributions are not sufficiently different, construct the CI about q by picking other "nearby" q values, computing their expected frequency distributions and accept or reject them using the G test.

For example, data for *Est-4*×*Est-3* can be regrouped into four hypothetical phenotype classes with A and B dominant. Using Steven's method (1939) based on phenotypes, our "best guess" of q is 0.145. For our case the frequency of each genotype (from left to right in Table 1) would be $Nq^2/4$, $Nq^2/4$, $Nq(1-q)/2$, $Nq(1-q)/2$, $Nq(1-q)/2$, $Nq(1-q)/2$, $Nq^2/2$, $N(1-q)^2/2$, $N(1-q)^2/4$, $N(1-q)^2/4$, where N = total number of progeny. Corresponding values are 1.06, 1.06, 12.46, 12.46, 12.46, 12.46, 2.11, 73.46, 36.73, 36.73. Because of small numbers, the first two classes are combined. By study design the 7th and 8th classes are combined. The G test of 8 classes gives a value of 7.740 and referring to a Chi Square table using 8-2 degrees of freedom gives $P>0.20$, so a q value of 0.145 cannot be rejected (with 95% confidence). An approximate confidence interval is determined by iteration, repeatedly testing over a range of q values. The 95% confidence interval is that set of q values not incompatible ($P<0.05$) with the observed data,

in this case 0.11–0.22. Furthermore, by using the q value corresponding to the maximum P value, representing the q value most compatible with the observed data, one can determine the "best fitting value" for q. For this case this occurs at q = 0.165 (P>0.30). Similarly the "best fitting value" for q and their 95% CI for the other pair combinations are as follows:

Est-4×*Est-1*	0.36 (P>0.50)	(0.28–0.49)
Est-3×*Pgd-2*	0.47 (P>0.20)	(0.38–0.50)
Est-4×*Pgd-2*	0.47 (P>0.99)	(0.36–0.50)
Est-1×*Pgd-2*	0.55 (P>0.30)	(0.44–0.50)
Est-3×*Est-1*	0.43 (P>0.20)	(0.35–0.50)

The esterase loci show linkage in the linear array *Est-1* – 36% – *Est-4* –16.5% – *Est-3*. Since these map distances cover more than 50% of the esterase linkage group, it is unlikely that the *Pgd-2* locus is in the same linkage group, over 50% recombination away from any of the esterase loci. Thus *Pgd-2* appears to be unlinked to any of the esterase loci.

Table 2 shows the frequencies of all the electromorphs seen in the colonies and the 'goodness of fit' of genotypes to those expected from the Hardy-Weinberg equilibrium formula.

Table 2. Frequencies of electromorphs within the colonies of the *Anopheles maculatus* used in this study

Colony		HK	KL
Sample size		90	80
Est-1	*103*	0.94	0.00
	100	0.05	0.60
	97	0.01	0.40
		P>0.90*	P>0.70*
Est-3	*103*	0.23	0.00
	107	0.19	0.00
	100	0.00	0.52
	98	0.08	0.00
	96	0.00	0.48
	95	0.49	0.00
		P>0.30	P>0.30
Est-4	*104*	0.94	0.00
	100	0.06	0.89
	'Null'	0.00	0.11
		P>0.50	

* are the P-values for tests of genotypic frequencies for 'goodness of fit' against those expected from the Hardy-Weinberg equilibrium formula.

Discussion

Designation of the esterase loci and their electromorphs follows that of Mahon et al. (1976) and Miles (1978). They used a similar TEB buffer system, at the same pH, but used starch gels. Comparison of their results to our Fig. 1A shows similar patterns between *A. gambiae* species and *A. maculatus,* suggesting that *Est-1* and *Est-3* are homologous loci between these two groups. Furthermore, *Est-3* might be homologous with *Est-alpha,* and *Est-4* with *Est-beta* in *A. culicifacies* (Dubash et al., 1982), based on the similarities in speed and substrate-specificity during staining. In the *A. culicifacies* work (*ibid*), *Est-1* did not appear; probably due to the short staining time (5–10 min vs. 2 h). *Est-1* in *A. maculatus* is revealed in the Poulik buffer system used in the *A. culicifacies* work (*ibid*), and we suspect that *Est-1* occurs in *A. culicifacies* because it occurs in closely related species. These are *A. minimus* (unpublished data), *A. funestus* (Green, 1977) and *A. marshallii* (Lambert, 1980).

Our immediate use for linkage information is in species studies of natural populations of *A. maculatus.* Often one finds that different genetic variants, including paracentric inversions and electromorphs, show linkage disequilibria phenomena in natural populations. If these persist through time then one might reasonably exclude the possibility of selective immigration/emigration, however, two equally possible hypotheses remain to account for the disequilibria. The variants are genetically linked or they characterise one of two or more unknown species within samples. In the absence of information about linkage from experimental crosses one cannot decide between these two hypotheses.

In determining recombination rates, traditional *Drosophila* formal genetics used mutants that usually were controlled by recessive alleles, where heterozygote and homozygotes for the dominant allele are confused in the single phenotype. In experiments following Mendel's own design of selfing the F_1 to obtain F_2 in dihybrid crosses, 50% of recombinant genotypes would be confused with parental genotypes. In order to estimate recombination and avoid this confusion, classical geneticists back-crossed the F_1 to the parental stock that was homozygous for the recessive mutants. In this way all recombinant chromosomes involving odd-numbered cross-overs, with respect to the markers, are identifiable from the phenotypes of the progeny of the back-cross. Typically anopheline geneticists have followed this simple design, e.g. the *A. culicifacies* work (Dubash et al., 1982). Anophelines present far greater logistic problems in the insectary than do *Drosophila,* e.g. they often will not mate on their own (the case with our *A. maculatus* stocks) and they need blood meals. Most insectaries cannot afford to establish the necessary homozygous stocks; indeed, sometimes this proves very difficult if not impossible with certain species even for sophisticated laboratories. We decided to use our unselected colonies and select appropriate families to provide recombination data. In fact progeny from wild-caught females could be used if variation is at a high enough frequency. Stevens (1939) provided a table in which estimates of linkage could be deduced from the

F_2 of selfed F_1 as long as one knew the parental chromosome types. His table (1939) was designed for the dominance/recessive situation. However in our case with co-dominant alleles, the problem is much reduced because only one sixth of the recombinant genotypes are confused with parental types, i.e. the double heterozygotes shown in columns 7 and 8, Table 1.

Our use of the dihybrid-cross method for estimating linkage is not as accurate as the traditional back-cross. To what degree the two estimates might differ awaits the appropriate test. However for our purposes, i.e. knowledge of linkage in analysing genetic data from natural populations, our estimates will serve. In any case exact measures of recombination derived by the back-cross method from highly-inbred laboratory stocks, cannot be taken as typical of species since mutants are known that influence the rate. In other words "exact" measures from highly-inbred laboratory stocks might have an "exact" value pertaining only to such stocks. The back-cross method does permit estimation of sex-specific recombination rates which the dihybrid-cross method does not permit and these rates may well differ. Our method gives an average of any such sexual difference. However, populations-genetic data would necessarily involve a mean of these two values so there is, in this case, no benefit to be derived from the added detail available from the back-cross method.

The likely polytene-chromosome/enzyme correlations for *A. maculatus* can be suggested through interspecific polytene chromosome homologies and known chromosome/enzymes correlations in other species. Esterases in *A. culicifacies* belong to linkage group 3 which is chromosome 3 composed of polytene arms 3R–3L (using the old arm designation and used by Dubach et al., 1982, or arms 4–3, respectively, in the more recent scheme of Green and Hunt, 1980). Furthermore a single esterase locus in *A. stephensi* belongs to linkage group 3 (accidently designated with the same number as group 3 in *A. culicifacies*) in that species (Iqbal et al., 1973) and has been correlated to polytene chromosomes (Sakai et al., 1983). Green and Hunt (1980) have established that these species and *A. maculatus* have the same association of polytene arms in their autosomes, i.e. 2–5 form one autosome and 3–4 form the other.

Green (1981) pointed out that since polytene chromosome homologies between species permitted an easy way to evaluate interspecific differences in genomic structure, at least for euchromatin, then interspecific homologies of gene linkage groups might well be inferred through interspecific homologies between polytene chromosomes. In other words the formal genetics of one species within the set of species for which polytene chromosome homologies were known could be generalised for the rest of the group. Homologies are known for *A. culicifacies* and species in the series Neocellia, of which *A. stephensi* and *A. maculatus* are members (Green, 1982a, b). The polytene chromosome elements implicated as locations of the esterase genes in both *A. culicifacies* and *A. stephensi* (Dubash et al., 1982 and Sakai et al., 1983) are in fact the same homologous polytene elements in these two species (Green, 1982a, b).

Furthermore polytene chromosome homologies suggest that *A. stephensi* and *A. maculatus* are immediate relatives within Neocellia (Green, 1982b; Green et al., 1985b). These species differ by one paracentric inversion in arm 3 and two paracentric inversions in arm 4 (Green, 1982b). It seems safe to assume that esterase loci in *A. maculatus* lie on the third chromosome, i.e. polytene arms 3 and 4. If indeed *Pgd-2* does not lie within the same linkage group then by elimination, it must lie on chromosome 2 (polytene arms 2–5 [Green and Hunt, 1980]). So far no floating inversions have been found on chromosome 3 in natural populations of *A. maculatus.*

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