MULTIPLE SEX DETERMINING REGIONS IN A GROUP OF RELATED *CHIRONOMUS* SPECIES (DIPTERA:CHIRONOMIDAE)

JON MARTIN, CHALIW KUVANGKADILOK, DIANNE H. PEART and BARRY T. O. LEE

Department of Genetics, University of Melbourne, Parkville Vic., 3052, Australia

Received 27.xi.79

SUMMARY

In a group of Australian *Chironomus* species, sex determining regions involved in male heterogamety have been found at a minimum of five different chromosomal locations. In one case, *Ch. oppositus* A, sex determining regions have been identified on two different chromosomes. It is uncertain whether this is a polymorphism or indicates the existence of multiple sex determining regions. It is argued that all the published data on sites of male determining regions are compatible with the five locations found in this study. On the basis of the postulated phylogeny of the studied group, the location of the sex determining regions would appear to have changed during speciation in all cases except one. The significance of these changes in location during the speciation process is discussed.

1. INTRODUCTION

CHIRONOMIDAE, in common with many other Diptera, do not in general have morphologically differentiated sex chromosomes. However, in the genus *Chironomus*, the sex linkage of certain inversions in *Ch. tentans* and *Ch. pallidivittatus*, as well as that of eye colour mutant in *Ch. pallidivittatus*, demonstrated that the males were the heterogametic sex (Beermann, 1955), the only exception being certain North American populations of *Ch. tentans* in which female heterogamety is indicated (Thompson, 1971). It was not possible to determine whether sex determination was due to a balance system as in *Drosophila* (Bridges, 1925) or to a dominant male determiner as in *Lucilia* (Ullerich, 1963).

The general location of sex determining regions has been established in a number of other species (table 5). These studies show that the sex determining region need not be in the same chromosome or chromosome arm (Beermann, 1955; Keyl, 1961; Rosin and Fischer, 1972) and that for some species, for example, *Ch. tentans* (Beermann, 1955; Thompson, 1971) *Ch. plumosus* (Acton, 1957; Keyl, 1961; Rosin and Fischer, 1972) and *Ch. nuditaris* (Rosin and Fischer, 1972) there may be more than one location for the sex determining region. Beerman (1955) interpreted his data on the basis that there was polymorphism for a Y chromosome.

In many *Chironomus* species, even though they may be highly polymorphic, there is no indication that sex linkage of inversions occurs in natural populations. In fact this would only be expected when the sex determining loci were actually included in the inverted region. If the sex determiners are linked to the inverted region and crossing-over can occur in the chromosome segment between them, the sex determining regions would be expected to be randomly distributed in natural populations unless selection or recent origin had lead to linkage disequilibrium (Geiringer 1944, 1948). Sex

linkage of inversion sequences or of individual loci should however be seen among the offspring of individual crosses. While individual crosses are difficult to perform with most *Chironomus* species in the laboratory, egg masses from single females can be relatively easily collected and the offspring reared. If it is assumed that the egg mass represents the result of a single mating, the offspring may be analysed to look for evidence of sex linkage in segregating inversions. Rosin and Fischer (1972) used this approach to locate sex determining regions in several European species. They point out that not all egg masses will provide useful information. Even if there are inversions segregating, only those crosses in which the male parent was heterozygous will be meaningful and the heterozygosity must be inferred from the distribution of inversions in the offspring when the parental karyotype is unknown. Despite these limitations, this technique is currently the only means of investigating sex determination in many species.

While European populations of *Ch. tentans* and *Ch. pallidivittatus* share a common sex determining region, although with differences in the complexity of the inversions involved (Beermann, 1955), a group of related Australian species, *Ch. oppositus*, *Ch. australis* and *Ch. duplex*, have the sex determining loci in different chromosomes (Martin, quoted in White, 1978). This is a common feature of many Simuliidae where it appears that the sex determining region is replaced during speciation (Rothfels, 1979; Bedo, 1975; White, 1978). The extent to which such replacement occurs in *Chironomus* species remains to be determined.

In this paper we report in more detail the sex determining regions of the three forms of *Ch. oppositus*, Forms A, B and C (Martin *et al.*, 1978) and three members of the *Ch. australis* group, *Ch. australis*, *Ch. duplex*, and *Ch. occidentalis* (Martin, 1971). This group of six taxa is relatively well defined cytologically although the specific status of the forms of *Ch. oppositus* has not yet been settled. Form B is relatively distinct from Forms A and C which can only be accurately separated on the basis of whether or not the inversion sequences in arm A show sex linkage in natural populations (Martin and Lee, 1980). In earlier studies only two species were recognised in the *Ch. australis* group (Martin, 1971). Subsequently material from Western Australia has been recognised as representing a distinct species with only three polytene chromosomes, as in *Ch. duplex*, but having a number of inversion differences (Martin, unpubl.) as well as differing in the number of nucleoli (Lentzios and Stocker, 1979). The Western Australian material has been called *Ch. occidentalis* and is considered to be a distinct species even though it will hybridise with *Ch. duplex* under laboratory conditions. The hybrids are fertile and can be used to investigate sex determination in these two species. Since the chromosomes of the hybrid are recognisable as to the species of origin, their presence in backcross progeny and in some cases whether the chromosome has undergone an exchange can be determined. It is of particular importance to be able to follow the Y chromosome of the original male parent. This technique can also be used with hybrids between some of the forms of *Ch. oppositus* although to a limited extent because of the difficulty in recognising the parental chromosomes in some hybrids (Martin and Lee, 1980).

Therefore, by a combination of techniques, we have been able to compare the location of sex determining regions in all members of this group and assess the importance of replacement of such regions in speciations.

2. MATERIALS AND METHODS

Most of the material used in this study has been either bred in the laboratory or obtained from wild collected egg masses subsequently reared in the laboratory. This necessitated the development of a culturing technique suitable for the Australian species. A variant of the medium developed for European species by Walter (1973) has been used, with the proportions of various ions adjusted to the high Na^{++} and Cl^- levels normally found in Australian waters (Bayly and Williams, 1973). Each salt was made up as a separate concentrate, 100 ml of concentrate containing: 1.0 g NaHCO_3 , 5.0 g NaCl , 1.0 g CaCl_2 , 0.2 g KH_2PO_4 , 1.0 g MgSO_4 and 1.8 g MgCl_2 respectively. The basic medium was obtained by adding 1 ml/l of each concentrate. This solution called 1X Martins, has Total Dissolved Solids (TDS) of 75 ppm. Solutions of higher TDS can be made by increasing the quantity of each concentrate to give 2X, 3X, etc. The medium was supplemented with either FeCl_3 or FeSO_4 , made up as a 0.1 per cent solution and added at the rate of 0.2 ml/l regardless of the concentration of other salts.

Egg masses were set up in individual containers, with a bottom area of about 625 cm^2 , in 4 l of solution. Three Kleenex industrial wipers, washed in absolute ethanol, were shredded and added to provide a substrate on which the larvae could build their tubes and feed. Additional tissues were added as necessary. Further food was provided in the form of Trout Starter 1C (supplied by Rural Chemical Industries) at the rate of 0.2 g initially and 0.4 g/week. This was supplemented by 1 ml of a solution containing 0.6 g Thiamine HCl/100 ml, given at the time of setting up the egg mass. Containers were aerated continuously with filtered air.

Egg masses or larvae of *Chironomus oppositus* were reared in 1X Martins; *Ch. australis*, *Ch. occidentalis* and *Ch. duplex*-*Ch. occidentalis* hybrids or backcrosses in 2X Martins; *Ch. duplex* in 3X or 4X Martins. Larvae for cytological analysis were removed at late fourth instar and sexed by examination of the ventral invaginations of the twelfth segment (Martin, 1962). Whenever possible the sexing was double checked to eliminate errors. Egg masses of the various forms of *Ch. oppositus* have come from a number of areas, particularly Bellerive and Queenstown, Tasmania; Anglesea, Mitcham and Albert Park in Victoria. *Ch. duplex* material has come from the Metropolitan Farm, Werribee, with some samples from Lake Learmonth near Ballarat, both in Victoria. *Ch. australis* egg masses were from several localities in Tasmania, Kimba, South Australia and Belconnen, A.C.T. The exact source of the *Ch. occidentalis* egg masses is not known but is probably in the vicinity of Perth, W.A.

Laboratory matings were carried out in cages 0.3 m^3 in a controlled temperature room (20°C) with a 16 hr light/8 hr dark cycle, including 1 hr artificial twilight at dawn and dusk. For *Ch. oppositus* crosses, at least 20 males were required (Martin and Lee, 1980), while for *Ch. duplex*, *Ch. occidentalis* or their hybrids only a few males were needed. *Ch. australis* has not been bred successfully in the laboratory. Egg masses were collected, checked for fertility and then reared in the same manner as wild collected masses.

The same basic protocol applied when using hybridisation to observe sex determining regions regardless of the species involved. This protocol

FIG. 1.—Schematic outline of the crosses involved in using hybridisation between *Chironomus occidentalis* and *Ch. duplex* to identify the Y chromosome of *Ch. occidentalis*. ♂-karyotype which will definitely be male. ♀-karyotype which will definitely be female. The sex of the other backcross karyotypes can only be determined by the analysis of the results of such crosses.

for *Ch. occidentalis* is shown in fig. 1. A male of the species under study (*Ch. occidentalis*) was mated to a female of another species or form (*Ch. duplex*). The hybrid male must therefore carry the male sex determining region of the original *Ch. occidentalis* male and this chromosome will pass only to male offspring in a backcross. The hybrid male may be backcrossed to females of either parent form but for simplicity of scoring the form of the original female parent was normally used (*Ch. duplex*). The only *Ch. occidentalis* chromosomes in the cross then are from the hybrid male, and the backcross larvae may be easily scored to determine which chromosome arm passes always to male offspring. In the case of backcrosses using the forms of *Ch. oppositus*, some arms cannot be scored since both parents may carry the same sequence.

3. RESULTS

The analyses of egg masses, whether wild caught or laid in the laboratory, are consistent with the assumption that even though the parents are unknown, they are the result of single insemination. Each species will be considered separately, the inversion terminology follows previous usage for *Chironomus oppositus* (Martin *et al.*, 1978) and *Ch. duplex* (Martin, 1971).

Chironomus oppositus Form A (*Ch. oppositus* A)

This form is highly polymorphic with inversions in all chromosome arms, but there is no indication of sex linkage associated with any inversion in the natural populations studied so far (Martin, 1969; Martin *et al.*, 1978). Egg masses have been investigated in which an inversion was segregating in arm B, F, D, C or A as the result of a homozygote $\times$ heterozygote mating. Inversions in arms F, D and C have shown evidence of sex linkage (table 1).

TABLE 1
Segregation of inversion sequences in arms F, D and C of *Ch. oppositus* Form A

Egg Mass		F ₁ /F ₁	F ₁ /F ₃	Total
Anglesea, Vic.				
Egg mass 210	Female	27	—	27
	Male	—	33	33
Egg mass 218	Female	28	—	28
	Male	—	28	28
Albert Park, Vic.				
Egg mass 3	Female	D ₁ /D ₁	D ₁ /D ₄	Total
	Male	—	26	26
	Female	20	—	20
	Male	—	—	—
Mitcham, Vic.				
Egg mass 2	Female	D ₁ /D ₁	D ₁ /D ₃	Total
	Male	67	1	68
Egg mass 7	Female	1	59	60
	Male	51	67	118
	Female	48	36	84
	Male	—	—	—
Melbourne Exhibition Gardens, Vic.				
Egg mass 4	Female	C ₂ /C ₂	C ₁ /C ₂	Total
	Male	25	1	26
	Female	—	26	26
	Male	—	—	—
Queenstown, Tas.				
Egg mass 2	Female	C ₁ /C ₁	C ₁ /C ₂	Total
	Male	12	9	21
	Female	5	1	6
	Male	—	—	—

Sequence F_3 is rarely found and only two egg masses segregating this sequence along with F_1 have been scored. In both, F_3 was segregated into the male larvae only.

Inversions in arms D and C are considered together because the results are basically similar. The inversions in these arms do not always show sex linkage (*e.g.*, Martin and Lee, 1980 and table 1). This is to be expected because only those egg masses in which the male parent was heterozygous would show evidence of sex linkage (Rosin and Fischer, 1972). Therefore egg masses showing no evidence of sex linkage would result from crosses where the female parent was heterozygous.

Among those egg masses which showed sex linkage, some produced virtually all heterozygous male and homozygous female larvae. In other cases the females were heterozygous and the males homozygous. The heterozygous male parent will carry one sequence in common with the homozygous female parent and a different sequence on the homologue. If the different sequence is on the Y chromosome the male offspring will be heterozygous, but if it is on the paternal X-chromosome then the female offspring will be heterozygous. The two arm D inversions scored, D_3 and D_4 , are about the same length so the results for the two egg masses involving these inversions were pooled and showed a recombination frequency of 1.2 per cent between the inversion and the sex determining loci. The small sample involving arm C showed a recombination frequency of about 2 per cent. The fact that inversions on either the D or C arm may show linkage and small amounts of recombination with the sex determining loci is best explained by assuming that this sex determining region is near the centromere of the DC chromosome. Therefore there would appear to be at least two sex determining regions in this form, one in arm F and the other near the CD centromere.

Ch. *oppositus* Form B (Ch. *oppositus* B)

There is no indication of sex linkage associated with an inversion sequence in natural populations of this form. Egg masses have been investigated in which inversions in arms B, F, C, A or G were segregating. Only an inversion in arm G showed evidence of sex linkage in some cases (table 2; Martin and Lee, 1980). Although arm G is relatively short, there is recombination between the small inversion and the sex determining region. The results (table 2) suggest that there is heterogeneity in the recombination frequency between different egg masses. The three generations derived from egg mass 26 show no significant heterogeneity but the frequency from all egg masses varied from less than 1 per cent to over 9 per cent of recombination. The egg mass with the highest frequency of recombination had three other inversions segregating while the egg mass with the lowest frequency had two other inversions, compared to the intermediate egg mass 26 series with only one other inversion segregating. This would suggest that recombination frequency is influenced by the genetic background, and may also depend on the proportion of the genome tied up in inversion heterozygosity, but these results would imply no direct correlation.

Ch. *oppositus* Form C (Ch. *oppositus* C)

Form C has been defined cytologically on the basis that arm A inversions show sex linkage in natural populations. This sex linkage has been con-

TABLE 2

Segregation of arm G sequences showing sex linkage in Ch. oppositus Form B from Bellerive, Tasmania

Egg mass		G ₁ /G ₁	G ₁ /G ₂	Total
9	Female	77	1	78
	Male	—	51	51
Recombination between inversion and sex region = 0.8%				
10	Female	94	7	101
	Male	8	55	63
Recombination between inversion and sex region = 9.2%				
26	Female	22	2	24
	Male	1	25	26
Recombination between inversion and sex region = 6.0%				
F ₁ of 26	Female	15	1	16
	Male	—	22	22
Recombination between inversion and sex region = 2.6%				
F ₂ of 26	Female	12	—	12
	Male	1	11	12
Recombination between inversion and sex region = 4.2%				
Recombination between inversion and sex region (all generations of egg mass 26) = 4.5%				
Homogeneity of recombination rates all egg masses.				
$\chi^2_2 = 8.51$ $P < 0.02$				

firmed from the segregation patterns in a number of egg masses and laboratory crosses of Form C males to Form B females. In most of the egg masses studied there was no recombination between the inversion and the sex determining region. From a total of 1080 larvae examined only four individuals, from three egg masses out of 55, showed recombination, a frequency of 0.37 per cent. However, since these egg masses were not double-checked for the sex of the larvae, it is possible that the small apparent recombination frequency simply reflects the frequency of misclassification and that the sex determination is completely linked with the inversions A₂ and A₃ which both cover much the same region (Martin, 1969; Cragg, unpubl.).

In addition to the arm A sequences, the segregation of inversions in arms B, D, C or E was investigated. Arm E shows evidence of sex linkage in some backcrosses of hybrid males, resulting from the cross of Form C males with Form B females, to females of either Form B or Form C. This is presumably linkage to the sex determining region in arm A, the other arm of chromosome III (Martin, 1969). In hybrids derived from Anglesea, Vic., recombination frequency between the sex determining region and inversion E₂ is about 30 per cent, while in hybrids derived from Bellerive, Tas., there is independent assortment of arm E sequence and sex.

One small egg mass sample, however, suggested sex-linkage of arm D sequence since, of the 12 females scored, nine were D₁/D₂ and three D₂/D₂, whereas in the 12 males, eight were D₁/D₁ and four D₁/D₂. This egg mass would appear to have resulted from a cross between two heterozygotes in which the male or even the female chromosomes were acting as a heterogametic pair. However, this result is probably due to chance since the egg mass was obtained from a laboratory cross of adults from two other egg masses which show no evidence of sex linkage in arm D. Also there is no

evidence of sex linkage of this arm in hybridisation crosses in which it is known that the male parent was heterozygous for D_1/D_2 .

Ch. australis

Although no inversions are known in this species, there is definite indication in natural populations that there is a sex determining region near the centromere of the CD chromosome. The presence of three heterochromatic bands near the centromere of the CD chromosome which are heterozygous in males but homozygous in females suggests that there is a sex determining region in this area. The sex linkage of these bands has been confirmed in egg mass material.

Ch. duplex

Some natural populations of this species show clear sex linkage of the Werribee (We) inversion in arm B (Martin, 1967, 1971). The examination of egg mass material has clearly confirmed the presence of a sex determiner in arm B, even in those populations which are homozygous for the Werribee sequence. Linkage between the inversion and the sex determining region is very tight with only a single confirmed recombinant being recorded, a cross-over frequency of 0.3 per cent. Analysis of larvae from the backcross of male hybrids, from the cross between *Ch. duplex* males $\times$ *Ch. occidentalis* females, to *Ch. duplex* also confirmed the presence of the *Ch. duplex* sex determining region on chromosome 1 (BF). All male offspring carried the *duplex* chromosome 1, as shown by the results of a typical backcross (table 3), or at least carried part of the *duplex* arm B if recombination had occurred. So far, 9 recombinants have been found amongst 167 backcross larvae examined.

There are two regions paired in arm B of the hybrids; region 1 from about 1 B2 to 1 D5 and region 2 from about 3 A1 to 1 D3 (fig. 2). On the basis of the We sequence (Martin, 1971), it is possible to recognise recombination in the region 1 A1 to about 1 B2; from about 1 D6 to 3 A1 and from about 3 B1 to the centromere if crossing over has occurred in either of the two

TABLE 3

Segregation of Chironomus duplex chromosomes in the offspring of a backcross involving a hybrid male from the cross Ch. duplex ♂ $\times$ Ch. occidentalis ♀ (DOO backcross No. 1)

Chromosomes of <i>Ch. duplex</i> origin	Sex of offspring	
	♂	♀
Chrom 1, 2 & 3	16	0
Chrom 1 & 2	6	0
Chrom 1 & 3	8*	0
Chrom 2 & 3	0	9
Chrom 1 only	11	0
Chrom 2 only	0	5
Chrom 3 only	0	11
None (i.e., all <i>Ch. occidentalis</i>)	0	11
Total	41	36

* Includes one individual with recombination in chromosome 1 (see text).

FIG. 2.—Arm B of a hybrid between *Chironomus duplex* and *Ch. occidentalis* showing the two regions of pairing when the *Ch. duplex* sequence is We. N-nucleolus of the *Ch. duplex* (We) sequence. Arrow indicates the centromere of the *Ch. occidentalis* homologue.

FIG. 3.—Arm E (left) and arm A (right) of a hybrid between *Chironomus duplex* and *Ch. occidentalis*. The *Ch. duplex* homologue carries the Oat sequence in arm A and the two regions of pairing with the *Ch. occidentalis* homologue are indicated. N-nucleolus of the *Ch. duplex* homologue. Arrows indicate the centromeres of the two homologues.

pairing regions. Three males were recombinant as a result of a crossover in pairing region 2, so that all the chromosome proximal to 1 D3 was *occidentalis*, the distal region, at least up to 3 A1, being *duplex*. Two other males were the result of a crossover in pairing region 1. These males carried the *occidentalis* sequence to at least 1 B2 and the *duplex* sequence from at least 1 D5 to the end of arm F. Two females resulted from recombination in region 1, with the *duplex* sequence at least to 1 B2 and then *occidentalis* from at least 1 D5 to the end of arm F. Another female resulted from recombination in region 2, with the *occidentalis* sequence at least to 3 A1 and the *duplex* sequence proximal to pairing region 2. Thus all males carried the region 1 D6 to 3 A1 plus all or at least part of pairing regions 1 and 2 of the *duplex* We sequence, while the females lacked this region. The sex determining region should therefore lie between the limits 1 D6 to 3 A1 of the We sequence. The occurrence of crossing over between the Werribee inversion and the sex determiners would suggest it is not actually in the region of the inversion, about 2 A1-2 D2, although the possibility that the recombination is the result of a rare double crossover cannot be excluded. It is possible that the sex determiners are in one or other of the other two pairing regions, but if this is the case recombinants of the "wrong" sex should be recovered when larger samples are analysed.

Arm F also shows some evidence of sex linkage in egg mass data, although there is no indication of this in the frequency of the Farm inversion in natural populations. It was not always known whether the male or the female parent was heterozygous in the egg mass data, nor was it always able to be deduced from the results due to the relatively high level of recombination between the inversion and the sex determining region. By pooling the data of those few crosses in which the male was known to be heterozygous plus any which showed a significant or at least a consistent (*e.g.*, deficiency of St^{Fa} female and Fa male) deviation from random assortment, a minimum estimate of 34 per cent recombination between the inversion on arm F and the sex determining region on arm B was obtained. Although some of these crosses constituted a three-point test cross, *i.e.*, heterogametic males heterozygous for both the Werribee and Farm inversions, no recombinants between the Werribee inversion and the sex determining region were recovered so there was no indication on which side of the Werribee inversion the sex determiners were located. Backcrosses of the hybrid males indicate strong sex linkage of arm F because the limited pairing results in a low frequency of recombination between the sex determining region and arm F (fig. 2). Neither the Metropolitan inversion on arm D, nor the Oatland inversion on arm A (Martin, 1971), show any indication of sex linkage in egg mass data. Chromosome II and III segregate randomly in hybrid backcrosses.

Ch. *occidentalis*

Only small samples from natural populations have been examined and few inversion heterozygotes were observed. The egg masses from which the laboratory stocks were derived showed no inversion polymorphism. The only data on the location of sex determining regions of this species therefore comes from backcrosses of hybrids derived from a *Ch. occidentalis* male. Typical results of such backcrosses are shown in table 4. Chromosome III (arms GEA) passes to males except in some recombinants. The other

chromosomes segregate equally to each sex. So far, a total of 11 recombinants for chromosome III have been observed amongst 136 backcross larvae. Pairing of the homologues in the hybrid male will be slightly different, depending on whether the *duplex* sequence is Standard (St^{Oat}) or Oatlands (Oat). In the polytene chromosomes of the hybrid there are two pairing regions. Region 1 is from about 14B to the middle of 14D, the limits at both ends being set by physical changes, a nucleolus in 14A and an inversion of the region distal to 14 D8. Study of the recombinants, however, indicates

TABLE 4
Segregation of Chironomus occidentalis chromosomes in the offspring of a backcross involving a hybrid male from the cross Ch. occidentalis ♂ × Ch. duplex ♀ (ODD backcross No. 1)

Chromosomes of <i>Ch. occidentalis</i> origin	Sex of offspring	
	♂	♀
Chrom 1, 2 & 3	9*	0
Chrom 1 & 2	0	4
Chrom 1 & 3	7	0
Chrom 2 & 3	3	1*
Chrom 1 only	0	11
Chrom 2 only	0	4
Chrom 3 only	8	0
None (<i>i.e.</i> , all <i>Ch. duplex</i>)	0	8
Total	27	28

* includes 1♂ and 1♀ showing recombination in chromosome 3.

that the nucleolus is not a physical block to pairing in meiosis since cross-overs occur up to region 13D. Region 1 is in the same relative position in both St^{Oat} and Oat. Region 2 is from about 15 C1 to 15 E7. In the case of St^{Oat} this segment is at the end of the chromosome while in Oat the region 15 B9 to 15 E1 is inserted between 15 E6 and 7 (Martin, 1971), so that pairing region 2 is about 18-20 bands from the end (figure 3). Recombination in region 2 was not relevant, so the results are independent of which *duplex* sequence was involved.

There were five males recombinant in chromosome III. All of these carried the *occidentalis* sequence for arms G and E and region 14A, a cross-over having occurred somewhere in the 14B-14D region, so that the distal part of the arm carried the *duplex* sequence. Recombinant females were of two types. Two of them carried the *occidentalis* sequence of arm G and up to region 13D of arm E and then the *duplex* sequence with the nucleolar organiser and arm A. The other four females were the reciprocal of the male recombinants, *i.e.*, *duplex* sequence from arm G to 14B-D, and then the distal end of arm A with the *occidentalis* sequence. These results would indicate that the sex determining region is near the centromere, in region 14A of arm A.

4. DISCUSSION

The results indicate that a minimum of five sex determining regions are present in this group of *Chironomus* taxa. Since these sex determining regions do occur at different locations it is of interest to assess the role of these changes in the speciation process.

In the case of *Chironomus oppositus* the specific status of the three forms has not yet been decided, although Form B is clearly distinguishable from Forms A and C (Martin and Lee, 1980). The present work indicates that the three forms differ in the location of the sex determining genes. This does not necessarily mean that they are distinct species, nor do our results preclude the possibility of other sex determining regions in these taxa. Two basic problems prevent the use of sex determining regions as a criterion for species differentiation in this group. First, there are two different sex determining regions amongst material considered to be Form A, one on Chromosome I and the other on Chromosome II. Secondly, the limitations of the techniques available have prevented us from determining how widespread the different regions are in distribution and whether they are ever present in the same individual. In other species, such as *Ch. tentans* (Beermann, 1955) and *Ch. nuditaris* (Rosin and Fischer, 1972), the presence of two sex determiners in the same population has been demonstrated.

Beermann (1955) interpreted the presence of more than one sex determining region (Y determiner) in the same species as a polymorphism. There is, however, at least one other possible explanation, namely, that they are components of a complex sex determining system, operative in all males but not normally recognisable because of the rarity of visible markers in natural populations. When the previous reports of sex determining regions in the genus *Chironomus* are examined (table 5), it would appear that the minimum of five loci or regions would account for not only our results but all the published observations. These loci would be on arm A in the vicinity of the centromere (*nuditaris*, *crassicaudatus*, *plumosus*, *oppositus* C and *occidentalis*) arm B near the telomere (*tentans*, *pallidivittatus*, and *duplex*); arm C near the centromere (east Canadian race of *tentans*, *plumosus*, *australis* and *oppositus* A); arm F possibly in region 16 of Keyl's (1962) map (*tentans*, *annularius*, *obtusidens*, *rempelii*, *luridus*, *stigmaterus*, *bernensis* and *oppositus* A); and on arm G (*nuditaris* and *oppositus* B).

The five sex determining regions can be derived from the data presented in table 5 if the following points are taken into account. In *Ch. occidentalis* the Y determiner is near the centromere, but in other species the location is not certain except that in *Ch. nuditaris* it is near the centromere of the AB chromosome (Rosin and Fischer, 1972). To assume that the *Ch. nuditaris* sex determiner is actually on arm B is inconsistent with the data from *Ch. tentans*, *Ch. pallidivittatus* and *Ch. duplex* where the sex determining regions are towards the telomere. Certainly the position of the sex determining regions will alter as the chromosomes are rearranged in speciation but the bands near the centromere regions are rarely affected (Keyl, 1962). Therefore it is more logical to assume that the *Ch. nuditaris* sex determiner is actually in arm A, a similar location to that of *Ch. occidentalis*. The Y determiner in arm B would then represent a second region. In three of the species with a sex determining region on the CD chromosome, *Ch. australis*, *Ch. plumosus*, *Ch. oppositus* A (table 5), it has been found to be near the centromere. In *Ch. australis* the heterochromatic bands associated with the sex determining region are actually in arm C and in Canadian male heterogametic *Ch. tentans* a Y locus has been identified in arm C (Acton, 1959; Thompson and Bowen, 1972). It could therefore be argued that all these loci are on arm C near the centromere and that there is no sex determining region in arm D.

The most commonly observed sex determining region is that in arm F,

TABLE 5

Listing of the position of the sex determining regions recorded in species of Chironomus and the means by which they were recognised

Chromosome Arm	Species	Location	Means of recognition	Author
A	<i>crassicaudatus</i>	outside inversion	complex inversion	15
	<i>nuditarsis</i>	near centromere	complex and simple inversions	11
	<i>occidentalis</i>	near centromere	species hybrid	16
	<i>oppositus</i> C	outside inversion	simple inversion	8
B	<i>plumosus</i>	outside inversion	complex inversion	1, 5
	<i>duplex</i>	distal to inversion	complex inversion, species hybrid	6, 16
	<i>nuditarsis</i>	near centromere	simple inversion	11
	<i>pallidivittatus</i>	at or near telomere or near centromere	complex inversion	3, 11
	<i>tentans</i> (European)	as for <i>pallidivittatus</i>	complex inversion	3, 11
	<i>australis</i>	near centromere	heterochromatic bands	16
C	<i>oppositus</i> A	near centromere	simple inversion	16
	<i>plumosus</i>	near centromere	simple inversion?	11
	<i>pallidivittatus</i>	in arm F	complex inversion with arm F	3
	<i>tentans</i> (European)	as <i>pallidivittatus</i>	complex inversion with arm F	3
	<i>tentans</i> (Canadian)	outside inversion	complex and simple inversion	2, 13
D	<i>australis</i>	near centromere (in arm C?)	heterochromatic bands	16
	<i>oppositus</i> A	near centromere	simple inversion	16
	<i>plumosus</i>	near centromere	simple inversion?	11
E	none recorded			
F	<i>luridus</i>	outside inversion	simple inversion	1
	<i>annularius</i>	at or near telomere or segment 16 in the inversion	complex inversion	3, 5
	<i>bernensis</i>	in the inversion?	simple inversion	11, 14
	<i>obtusidens</i>	in the inversion?	simple inversion with heterochromatic band	4, 5
	<i>oppositus</i> A	outside inversion?	simple inversion	16
	<i>pallidivittatus</i>	at or near telomere or segment 16 in the inversion	complex inversion	3
	<i>tentans</i> (European)	as <i>pallidivittatus</i>	complex inversion	3
	<i>tentans</i> (east Canadian)	♀ determiner near telomere	complex inversion	12, 13
	<i>rempelii</i>	in the inversion?	simple inversion with puffed band	10
	<i>stigmaterus</i>	in the inversion?	simple inversion with puffed band	9
	<i>nuditarsis</i>	at distal end	simple inversion and heterochromatic band	11
G	<i>oppositus</i> B	outside inversion	simple inversion	7, 16

Key to list of authors: 1-Acton 1957, 2-Acton 1959, 3-Beermann 1955, 4-Keyl 1961, 5-Keyl 1962, 6-Martin 1971, 7-Martin & Lee 1980, 8-Martin *et al.* 1978, 9-Martin & Wülker 1974, 10-Rempel *et al.* 1962, 11-Rosin & Fischer 1972, 12-Thompson 1971, 13-Thompson & Bowen 1972, 14-Wülker & Klötzli 1973, 15-Wülker & Martin 1971, 16-present study.

which Keyl (1962) suggests is in segment 16 of his map of this arm. Most of the cases listed in table 5 are compatible with this interpretation except *Ch. stigmaterus*, where the apparently closely sex linked inversion in segments 5-6a (Martin and Wülker, 1974) is some distance from segment 16. However, as there is no information on recombination in this species, the possibility of the Y determiner being in segment 16 cannot be ruled out. The presence of a sex linked inversion in *Ch. luridus* which splits segment 16 (Keyl, 1962) suggests that the proposed location of the sex determining region can be narrowed down to the bands 16f-g, which are included in the inversion. On the other hand, the female heterogametic sex determiner found in the eastern Canadian race of *Ch. tentans* (Thompson, 1971), which is on arm F, could not be located in segment 16 which is near the centre of the arm since Thompson and Bowen (1972) state that it is near the end of the arm. This female sex determiner may represent a sixth sex determining region.

Since arm G is very much altered between species, there is no reason why the locus in *Ch. nudatarsis* and *Ch. oppositus* B should appear to be in the same relative position in the arm. However, a distal location of the region in both species would be consistent with the existing data.

The significance of the five regions is not clear at present. It may be that only one region is functional in any individual or alternatively that all regions are functional. There are two lines of evidence from our results, which suggest that the latter is not correct. First, inversions in one species covering approximately the same region as those which show sex linkage in other species show no indication of sex linkage in any egg mass data. For example, inversion B₂ of *Ch. oppositus* shows no indication of sex linkage in any of the forms although it covers the region 2 A1-2 D2 in which the *Ch. duplex* sex determiner was localised. Similarly, the arm A sequences of *Ch. oppositus* A and B, as well as *Ch. duplex*, do not show sex linkage, while those of *Ch. oppositus* C do, yet the same sequences are present in Form A (A₂ and A₅) and Form B (A₂). Secondly, in backcrosses of *Ch. duplex* × *Ch. occidentalis* and *Ch. oppositus* C × B hybrids, only one chromosome from the original parent male segregated preferentially with regard to sex, the others segregated randomly. This evidence is not conclusive since crosses involving *Ch. oppositus* A, which is known to have sex determining regions in two different chromosomes, have not been successful. Such crosses would show definitely whether only one of the two sex determining regions is operative in any male. The results of the crosses so far made do not rule out the possibility that there are loci concerned with sex determination at all sites, but only one site is male determining.

In the group we have studied it is found that each of the five postulated sex-determining regions is the functional male determiner in at least one taxon. To understand the significance of this observation, some understanding of the phylogeny of the group is necessary. The phylogenetic relationships of some members of the group can be represented as follows (Martin, 1971; Martin and Lee, 1980):

Based on this phylogeny we may assume that the CD sex determiner found in *Ch. australis* is the same as that in *Ch. oppositus* A, with the heterochromatinisation of the *Ch. australis* X chromosome occurring after speciation had occurred. With this exception, the other species all show a change in the location of the sex-determining region either during or following speciation. The phylogenetic distance between *Ch. oppositus* C and *Ch. occidentalis* makes it likely that the presence of a sex determining region in arm A of each taxon is the result of an independent event rather than derivation from a common ancestor. These changes in location between taxa can be compared to the situation in the Simuliidae which appear to have a similar sex determining mechanism. Vajime and Dunbar (1975) state that no two species of simuliid share an identical set of sex chromosomes. However, in many cases this refers to a sex determining region of similar location but identified by different inversions of heterochromatic bands (Bedo, 1975; 1979). Under this broad definition none of the *Chironomus* species studied would share the same sex determining mechanism, because although the sex determining regions may be in the same location they are associated with different inversions or cytological markers. These differences between species may be of similar significance in speciation to the differences between autosomal inversion polymorphisms but implies no change in the sex determining mechanism.

The presence of polymorphic sex determiners in *Ch. oppositus* A as well as in a number of other species suggests that changes in the sex determining region occur due to the fixation of the alternative regions in different daughter species. The fact that *Ch. oppositus* A is presently polymorphic for sex determiners would suggest that this species may be capable of further speciation, raising the question of whether the change in the location of sex determination regions in speciation is incidental or a cause of speciation. Beermann (1955) noted that in the presence of polymorphic Y determiners the male population was split up in terms of sex determination since there was no way in which a Y determiner could transfer to a male carrying the alternative Y. Several authors have noted the tendency in both chironomids and simuliids, for the accumulation of inversions, or other mechanisms to reduce crossing over, in the vicinity of the sex determining regions (*e.g.*, Beermann, 1955; Rothfels, 1956; Bedo, 1975, 1979). This could mean that a fairly large segment of the chromosome is tied to the sex determiner and hence incapable of transmission to males carrying an alternative Y chromosome. It may then be that the genes associated with the alternative Y chromosomes could adapt the males to different environments. One can then ask whether such a situation would ultimately be sufficient to subdivide the female population, perhaps aided by assortative mating or some other mechanism, to lead eventually to distinct species with different sex determining mechanisms. Computer simulation of this model is possible and may show whether this mode of speciation is theoretically feasible.

Acknowledgment.—This work was supported by Grants D71/17815, D1-77/15599 and D1-78/15465 from the Australian Research Grants Committee. We are grateful to the M.M.B.W. for permission to collect at the Metropolitan Farm, Werribee, to Dr D. H. D. Edward (University of Western Australia), Dr D. L. Porter and Mr G. Lentzios (both University of Melbourne) for provision of material and to Mss M. Sawyer, E. Harts and E. Marles for technical assistance.

5. REFERENCES

- ACTON, A. B. 1957. Sex chromosome inversions in *Chironomus*. *Am. Naturalist*, 91, 57-59.
- ACTON, A. B. 1959. A study of the differences between widely separated populations of *Chironomus (Tendipes) tentans* (Diptera). *Proc. R. Soc. Lond.*, 151B, 277-296.
- BAYLY, I. A. E., AND WILLIAMS, W. D. 1973. *Inland Waters and their Ecology*. Longman, Australia.
- BEDO, D. G. 1975. Polytene chromosomes of three species of black-flies in the *Simulium pictipes* group (Diptera: Simuliidae). *Canad. J. Zool.*, 53, 1147-1164.
- BEDO, D. G. 1979. Band and nucleolar polymorphisms in polytene chromosomes of *Simulium ornatis* (Diptera: Simuliidae). *Cytobios*, 21, 113-133.
- BEERMANN, W. 1955. Geschlechtsbestimmung und Evolution der genetischen Y-chromosomen bei *Chironomus*. *Biol. Zbl.*, 74, 525-544.
- BRIDGES, C. B. 1925. Sex in relation to chromosomes and genes. *Am. Naturalist*, 59, 127-137.
- GEIRINGER, H. 1944. On the probability theory of linkage in Mendelian heredity. *Ann. Math. Stat.*, 15, 25-27.
- GEIRINGER, H. 1948. On the mathematics of random mating in case of different recombination values for males and females. *Genetics*, 33, 548-564.
- KEYL, H.-G. 1961. Chromosomenevolution bei *Chironomus* I. Strukturabwandlungen an speicheldrüsen-chromosomen. *Chromosoma*, 12, 26-47.
- KEYL, H.-G. 1962. Chromosomenevolution bei *Chironomus* II. Chromosomenumbauten und phylogenetische Beziehungen der Arten. *Chromosoma*, 13, 464-514.
- LENTZIOS, G., AND STOCKER, A. J. 1979. Nucleolar relationships in some Australian *Chironomus* species as shown by Ag-As banding and other cytological techniques. *Chromosoma*, 75, 235-258.
- MARTIN, J. 1962. Interrelation of inversion systems in the midge *Chironomus intortinctus* (Diptera: Nematocera). I. A sex-linked inversion. *Aust. J. Biol. Sci.*, 15, 666-673.
- MARTIN, J. 1967. Meiosis in inversion heterozygotes in Chironomidae. *Canad. J. Genet. Cytol.*, 9, 255-268.
- MARTIN, J. 1969. The salivary gland chromosomes of *Chironomus oppositus* Walker (Diptera: Nematocera). *Aust. J. Zool.*, 17, 473-486.
- MARTIN, J. 1971. A review of the genus *Chironomus* (Diptera, Chironomidae). IV. The karyosystematics of the *australis* group in Australia. *Chromosoma*, 35, 418-430.
- MARTIN, J., AND LEE, B. T. O. 1980. Problems in speciation of *Chironomus oppositus* in south-eastern Australia. In *Essays on Evolution and Speciation in Honor of M. J. D. White*, ed. W. R. Atchley and D. S. Woodruff. Cambridge University Press, London.
- MARTIN, J., LEE, B. T. O., AND CONNOR, E. 1978. Apparent incipient speciation in the midge *Chironomus oppositus* Walker (Diptera: Chironomidae). *Aust. J. Zool.*, 26, 323-329.
- MARTIN, J., AND WÜLKER, W. 1974. A review of the genus *Chironomus* (Diptera, Chironomidae). VIII. The cytology of *Chironomus stigmaterus* Say. *Stud. Nat. Sci. (Portales, N. M.)*, 1, (11), 1-17.
- MARTIN, J., WÜLKER, W., AND SUBLETTE, J. E. 1974. Evolutionary cytology in the genus *Chironomus* Meigen. *Stud. Nat. Sci. (Portales, N. M.)*, 1, (12), 1-12.
- REMPEL, J. G., NAYLOR, J. M., ROTHFELS, K., AND OTTONEN, B. 1962. The sex chromosome constitution of Chironomid intersexes parasitised by Nematodes. *Canad. J. Genet. Cyt.*, 4, 92-96.
- ROSIN, S., AND FISCHER, J. 1972. Polymorphismus des realisators für männliches Geschlecht bei *Chironomus*. *Rev. Suisse Zool.*, 79, 119-141.
- ROTHFELS, K. H. 1956. Black flies: siblings, sex and species grouping. *J. Heredity*, 47, 113-122.
- ROTHFELS, K. H. 1979. Cytotaxonomy of black flies (Simuliidae). *Ann. Rev. Entomol.*, 24, 5-539.
- THOMPSON, P. E. 1971. Male and female heterogamety in populations of *Chironomus tentans* (Diptera: Chironomidae). *Canad. Entomologist*, 103, 369-372.
- THOMPSON, P. E., AND BOWEN, J. S. 1972. Interactions of differentiated primary sex factors in *Chironomus tentans*. *Genetics*, 70, 491-493.
- ULLERICH, F. 1963. Geschlechtschromosomen und Geschlechtsbestimmung bei einigen Calliphorinen (Calliphoridae, Diptera). *Chromosoma*, 14, 45-110.
- VAJIME, C. G., AND DUNBAR, R. W. 1975. Chromosomal identification of eight species of the subgenus *Edwardsellum* near and including *Simulium (Edwardsellum) damnosum* Theobald (Diptera: Simuliidae). *Tropenmed. Parasit.*, 26, 111-138.

- WALTER, L. 1973. Syntheseprozesse an den Riesenchromosomen von *Glyptotendipes*. *Chromosoma*, 41, 327-360.
- WHITE, M. J. D. 1978. *Modes of speciation*. *Books in Biology Series*, ed. C. I. Davern. Freeman and Co., San Francisco.
- WÜLKER, W., AND KLÖTZLI, A. M. 1973. Revision der Gattung *Chironomus* Meig. IV. Arten des *lacunarius*-(*commutatus*-) Komplexes. *Arch. Hydrobiol.*, 72, 474-489.
- WÜLKER, W. F., AND MARTIN, J. 1971. C. Karyosystematics of the *Chironomus staegeri* group. *Stud. Nat. Sci. (Portales, N. M.)*, 1, (1), 22-34.