Sex chromosome evolution in *Simulium erythrocephalum* (Diptera: Simuliidae)

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The sex chromosomes of *Simulium erythrocephalum* show a pattern of serial differentiation by the linkage of inversions to either X or Y. Two inversion systems, *IL-A* and *IS-B + I + G* are maintained in stable polymorphism with sex differences in frequency throughout southern England. These inversions are linked to the sex chromosomes such that males are more likely to be heterozygous. Heterozygosity and homozygosity for inversions *IL-A* and *IS-B* respectively seem to be associated with speed of larval development and are probably selected.

INTRODUCTION

Sex chromosome heteromorphy is known from many groups of animals and plants, and in most cases it is the Y-chromosome which is the smaller element. Presumably such systems evolved from morphologically identical and freely recombining sex chromosomes. Muller (1914) envisaged this process as a consequence of the perpetual heterozygosity of the Y-chromosome, implying that it alone was able to degenerate by the accumulation of recessive deleterious mutations, such as small deletions and other gene dysfunctions, because these will be masked by the equivalent functional locus on the X-chromosome. Specific numerical models of this process assume that recombination is already suppressed between the X and Y (Nei, 1970; Charlesworth, 1978). Thus attention focuses on mechanisms which restrict recombination between the sex chromosomes, and structural rearrangement by inversion is one such mechanism.

Size difference between the sex chromosomes of *Simuliidae* is virtually unknown, and in most species they are indistinguishable in the polytene nucleus. Furthermore, males are usually chiasmate (Rothfels and Mason, 1975) and it is therefore probable that sex is determined primarily by a single locus, or at most a very short length of chromosome. However, many studies have revealed species in which the sex chromosomes

have become differentiated by the linkage of inversions. Such inversions may be fixed or polymorphic on either of the sex chromosomes, or occur on both but at different frequencies (Post 1982a). The purpose of this article is to describe the behaviour of several such inversions in the blackfly *Simulium erythrocephalum*, and to attempt to identify the selective forces which act upon them and hence might lead to the restriction of recombination between the X and Y.

MATERIALS AND METHODS

Simulium (Boophthora) erythrocephalum (De Geer, 1776) is a small blood-sucking fly with immature stages restricted to rivers where the larvae are filter feeders. In central Europe *S. erythrocephalum* can be a serious agricultural pest and man-biter. Although it is also common in certain lowland British rivers, here it never seems to present much of a nuisance (Post, 1982b).

Post (1983) presented size frequency histograms for a series of regular samples of larvae taken from the River Tiffey at Carlton Forehoe in Norfolk during 1978. It is possible not only to identify each of the five annual generations from these histograms, but also to separate samples of larvae attaining their last instar earlier or later than the majority within that generation. A number of other rivers in southern England were occasionally sampled, and collection data are presented in table 1.

Larvae were fixed in the field in freshly mixed Carnoy's solution and stored at 0°C. Only large

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Table 1 Sample data and chromosome frequencies

Collection No.	Site	OS grid ref.	Date	Generation ¹	Chromosomes observed ²										
					IL					IS					
					A	St ³	B.I.G	I.G	B.G	B	I	G	St	B.I	
13876	R. Tiffey at Carlton Forehoe, Norfolk	TG 092 053	13.8.76	—	11	13			2	5	3	1	7		
16977			16.9.77	—	14	26		15		13		5	7		
411771			4.11.77	—	10	26		12	3		2	4	9		
30178			30.1.78	Early First	14	32		10	2	13	1	7	13		
22278			22.2.78	Early First	21	37		21		16	1	5	19		
27378			27.3.78	Early First	17	27		12	3	13	2	6	13		
10478			10.4.78	Late First	22	38		16		25	2	5	12		
5578			5.5.78	Late First	0	2		1					1		
9678			9.6.78	Early Second	18	48		19	1	18	2	6	20		
20678			20.6.78	Late Second	20	26		10	1	19	2	4	10		
27678			27.6.78	Early Third	32	40		12	1	22	2	5	28		
3778			3.7.78	Late Third	32	50		23		25	1	8	25		
89781			8.9.78	Late Fifth	36	36		15		28	1	8	26		
281077 ⁴	R. Yare at Bawburgh, Norfolk	TG 157 087	28.10.77	—	10	22		6		8	3	4	5		
411772	R. Blackwater at Hingham, Norfolk	TG 023 050	4.11.77	—	22	22		8	1	13	2	2	22		
89782	R. Wensum at Lenwade, Norfolk	TG 108 187	8.9.78	—	27	41		10	1	33		3	21		
61177	R. Little Ouse at Knettishall Heath, Norfolk	TL 955 807	6.11.77	—	10	14		5	6	13	5	6	15		
13878	R. Wey at Jacobs Well, Surrey	TQ 005 531	13.8.78	—	32	30		6	6	28		2	20		
1978 ⁵	R. Stour at Spettisbury, Dorset	ST 919 026	1.9.78	—	25	23			1	25		1	21		

1. See Post (1983).

2. The number of animals scored for *IL* is not always the same as for *IS* because it was not always possible to determine *IS* linkage arrangements.3. *St* signifies the arbitrarily chosen "standard" chromosome sequence and rare derivative sequences.

4. Collected as small larvae and reared to last instar using the laboratory technique described by Post (1982).

5. Collected by J. Bass, Freshwater Biological Association.

larvae (last or penultimate instar) were used for chromosome preparation. Larvae were split open along the ventral surface of the abdomen and the silk glands removed (see Puri, 1925). The silk glands were softened, on a microscope slide, with a few drops of 80 per cent acetic acid for five minutes and then stained in lacto-propionic orcein for four minutes. The silk glands were washed in 60 per cent acetic acid and the epithelium peeled off the jelly-like gland contents. The epithelium was restained for four minutes in lacto-propionic orcein and squashed in 60 per cent acetic acid. The preparation was made permanent, after examination, by the freeze-dry technique using euparal (Darlington and La Cour, 1976).

Whenever possible the larval body was subsequently stained with Feulgen (Darlington and La Cour 1976) to reveal the gonadal rudiment. The shape of the gonadal rudiment is a very reliable indicator of sex (Puri 1925), but in some larvae the rudiment could not be observed, including the whole of samples 55781 and 89782. This was usually the result of technical problems in dissection and staining, but also the rudiments can be underdeveloped in parasitized larvae. Feulgen stained testes were squashed and mounted in 60 per cent acetic acid for observation of male meiosis.

GENERAL KARYOTYPE

Testicular meiosis was found to be chiasmate (fig. 1) demonstrating that recombination occurs in males of *S. erythrocephalum*, as it does in most species of blackfly (Rothfels and Mason 1975).

The haploid complement of *S. erythrocephalum* is $n=3$, which is also the number of elements observed in the polytene nucleus, because synapsis of the homologues is almost complete (fig. 2).

From a total of 510 specimens sampled from seven sites (table 1) 34 obvious chromosomal rearrangements were observed across all six chromosome arms, but the only rearrangements to show any sex linkage are some of those on the first (longest) chromosome. It is concluded therefore that it is this chromosome which forms the sex chromosome pair. The observed sex-linked rearrangements can be separated into two classes: those which are limited to one sex chromosome or the other (including *IL-Q.R.S.T* and *IS-P+H*), and those which occur on both X and Y but at different frequencies (including *IL-A* and *IS-B+I+G*).

SEX CHROMOSOME DIFFERENTIATION

On examination of the polytene chromosomes from the larval silk glands all males were found to possess a short non-pairing region at the base of chromosome arm *IS* (and here labelled *IS-split*: fig. 3). Furthermore one of the bands within this non-pairing region was consistently heterozygous for intensity of staining. In females this band was homozygous for the fainter staining condition, and the bivalent was fully synapsed throughout its length, with a single exception. One female was found to possess *IS-split*, but it could not be determined if this was also associated with heterozygosity for the band staining. Band heteromorphy is not uncommon in blackflies and has previously been found associated with sex (Bedo, 1978).

The complex rearrangement labelled *IL-Q.R.S.T* was found 16 times, but only as the heterozygote, and only in males from Norfolk. None of the individual inversions which make up *IL-Q.R.S.T* were found independently. The constituent inversions *IL-Q* and *R* are distal, non-overlapping, and distinctive. However, the basal overlapping part of the complex forms a tangled heterozygous loop which varies between individuals, and it is not clear whether this is due to variation in the number of inversions linked in the complex, or variation in the sequence of the other homologue. However, the basal overlapping complex consists of at least two inversions (*IL-S* and *T*) and probably more. It is possible that *IL-A* might be included. *IL-Q.R.S.T* is shown in fig. 4.

The inversion *IS-P* (fig. 5) was found in six larvae taken from Carlton Forehoe, and also in one additional larva from Hingham. The inversion *IS-H* (fig. 5) was found in a total of four larvae taken from Carlton Forehoe and Hingham. On each occurrence *IS-H* was linked to *IS-P*. Neither inversion was found in the homozygous state, and all seven larvae were males which also possessed the heterozygous complex *IL-Q.R.S.T*.

It is concluded that *IS-split* is due to genetic differences between the X and Y chromosomes and represents the primary differential region of the sex chromosomes, including or very close to the primary sex determining locus. The single heterozygous female is enigmatic, but could result from an exceptional crossover between *IS-split* and the primary sex determining locus, or be the result of some rare mutation. Alternatively, one can never entirely rule out the possibility that specimens might have been mislabelled. *IL-Q.R.S.T* appears to mark the Y chromosome. Its distribution between males and females (16:0) is

Figure 1 Testicular meiosis from last larval instar *S. erythrocephalum*.

Figure 2 Entire polytene karyotype from female larval salivary gland.

Figure 3 *IS-B/St* from male, showing basal non-pairing region including band-staining dimorphism.

Figure 4 *IL-Q,R,S,T/St* from male *S. erythrocephalum*.

Figure 5 *IS-P.H/St* from male *S. erythrocephalum*.

Figure 6 *IL-A/St* from male.

Figure 7 *IS-G.I/B* from male.

significantly different from the observed Norfolk sex ratio (157:200) and the observed sex distribution of *IL-A* (155:103, see below). If *IL-Q.R.S.T* marks the Y-chromosome, then, presumably, *IS-P* and *IS-H* do also.

Thus, there seems to be a pattern of serial differentiation of the sex bivalent (fig. 8). *IS-split* represents the primary differential region, including, or at least very close to the primary sex determining gene. The linkage of *IL-Q.R.S.T*, *IS-P* and

Figure 8 Idiograms of *S. erythrocephalum* first-chromosome showing sex-chromosome differentiation.

IS-H to the Y-chromosome results in the serial elimination of recombination along the sex chromosomes out from the primary sex determining locus, thus effectively increasing the size of the sex chromosome differential region.

THE GENETICS OF INVERSION *IL-A*

The polymorphic inversion *IL-A* (fig. 6) was found at all seven sample sites (table 1) and its frequency did not vary geographically ($\chi^2_6 = 10.19$, $P = 0.12$). Furthermore, there is no evidence for temporal variation in inversion frequency between the 13 samples from Carlton Forehoe (combining samples 10478 and 5578; $\chi^2_{11} = 13.24$, $P = 0.28$). However, considering all 17 sexed samples, in 15 *IL-A* is at a higher frequency in males than females (data not shown) and this is significantly different from the random expectation of 8.5 ($\chi^2_1 = 9.94$, $P < 0.01$).

The karyotype frequency distribution in the two sexes at Carlton Forehoe during 1978 (table 2) shows a general excess of heterozygotes compared with Hardy-Weinberg expectation, although this is rarely significant in individual samples with the numbers obtained. However, considering the total data (table 2) there is a significant excess of male heterozygotes ($\chi^2_2 = 14.81$, $P < 0.001$), but not females ($\chi^2_2 = 1.40$, $P > 0.20$), and this excess occurs only in males from "early" samples ($\chi^2_2 =$

Table 2 Karyotype frequency data for Carlton Forehoe

Collection	Sex ¹	IL Karyotype			IS Karyotype																				
		A/A	A/St	St/St	I/G/I/G	I/G/B/G	I/G/B	I/G/I	I/G/G	I/G/St	B.G./B.G	B.G/B	B.G/I	B.G/G	B.G/St	B/B	B/I	B/G	B/St	I/I	I/G	I/St	G/G	G/St	St/St
30178	M	2	6	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	3	0	0	0	0	2	1
	F	0	4	11	1	1	2	1	2	0	0	1	0	0	0	0	0	1	4	0	0	0	0	1	0
22278	M	2	10	1	1	0	1	1	0	4	0	0	0	0	0	0	0	1	4	0	0	0	0	1	2
	F	0	6	9	3	0	4	0	1	2	0	0	0	0	0	1	0	1	2	0	0	0	0	1	0
27378	M	0	8	2	0	0	0	0	1	4	0	0	1	0	0	0	0	1	4	0	0	0	1	0	1
	F	1	7	4	1	1	1	1	0	1	0	0	1	0	0	3	0	0	0	0	0	0	0	1	1
10478	M	4	2	1	0	0	2	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1
	F	1	8	11	2	0	5	0	0	2	0	0	0	0	0	5	2	1	2	0	0	0	0	1	0
9678	M	0	10	1	0	0	1	0	0	3	0	0	0	0	0	1	0	1	2	0	0	2	0	1	0
	F	1	5	15	1	0	6	0	2	3	0	0	0	0	1	1	0	0	2	0	1	1	0	2	1
20678	M	3	7	1	0	0	1	0	1	2	0	0	0	0	1	0	1	1	1	0	0	1	0	0	1
	F	1	5	6	0	0	4	0	0	2	0	0	0	0	0	5	0	0	1	0	0	0	1	0	0
27678	M	3	13	1	1	0	2	0	1	2	0	0	0	1	0	0	0	0	6	0	0	0	0	1	3
	F	2	9	8	0	0	2	0	0	3	0	0	0	0	0	1	2	8	0	1	0	0	0	0	1
3778	M	5	10	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	5	0	0	0	1	3	1
	F	0	11	13	4	0	4	0	1	4	0	0	0	0	0	4	0	1	3	0	0	0	0	1	2
89781	M	10	10	3	0	0	3	0	1	6	0	0	0	0	0	0	2	9	0	0	1	0	1	3	
	F	0	5	7	0	0	4	0	0	1	0	0	0	0	0	2	0	4	0	0	0	0	0	0	1
All "early" samples	M	7	47	5	2	0	5	1	2	14	0	0	1	1	0	1	0	4	19	0	0	2	1	5	7
	F	4	31	47	6	2	15	2	5	9	0	1	1	0	1	5	1	4	16	0	2	1	0	5	3
All "late" samples	M	22	29	5	1	0	7	0	2	10	0	0	0	0	1	1	2	4	16	0	0	2	1	5	6
	F	2	29	37	6	0	17	0	1	9	0	0	0	0	0	16	2	6	6	0	0	0	1	2	3
Total	M	29	76	10	3	0	12	1	4	24	0	0	1	1	1	2	2	8	35	0	0	4	2	10	13
	F	6	60	84	12	2	32	2	6	18	0	1	1	0	1	21	3	10	22	0	2	1	1	7	6

1. It was not always possible to determine larval sex. Such animals are included in table 1 but not table 2.

20.89, $P < 0.001$) and not males from "late" samples ($\chi^2_2 = 1.11$, $P > 0.20$).

The excess of heterozygotes and higher frequency of *IL-A* in males could result from a higher frequency of *IL-A* on the Y-chromosome, as opposed to the X-chromosome. The frequency of *IL-A* on the X-chromosome (P_{xA}) is given by the frequency in females; and the frequency on the Y-chromosome, P_{yA} = Twice the frequency in males— P_{xA} . The expected male karyotype frequency distribution for each generation, calculated using these sex-chromosome inversion frequencies, is a much better fit than is obtained by assuming equal frequencies on the X and Y chromosomes (table 3), except for the fifth generation; but this might be expected, because the fifth generation is only represented by a single "late" sample.

To summarise, there is evidence that heterozygosity for inversion *IL-A* is correlated with faster larval development of males, but not necessarily females. Furthermore, *IL-A* occurs at a higher frequency on the Y-chromosome, as opposed to the X chromosome, and this results in an overall excess of male heterozygotes.

THE GENETICS OF *IS-B.I.G*

The polymorphic inversion *IS-I* is tandem and included in *IS-B*, which is proximal and does not overlap the distal polymorphic inversion *IS-G* (see figs. 7 and 8). However, these inversions were found in linkage disequilibrium. The chromosome sequences *IS-B.I* and *IS-B.I.G* were not found at

all, and inspection of Table 1 indicates a deficiency of *IS-B.G*.

The sample frequencies of the *IS* chromosome sequences (table 1) reveal no evidence for temporal variation between the 13 samples from Carlton Forehoe (combining samples 10478 and 5578, and sequences *B.G*, *I* and *St*; $\chi^2_{33} = 32.70$, $P = 0.5$), but suggest geographic variation between the seven sites (using total Carlton Forehoe data and combining sequences *B.G*, *I* and *St*; $\chi^2_{18} = 58.33$, $P < 0.0001$). Furthermore, in all 17 sexed samples *IS-St* was at higher frequency in males than females (data not shown), and this is significantly different from the random expectation of 8.5 ($\chi^2_1 = 8.5$, $P < 0.001$). Similarly, *IS-B* was found at higher frequency in 13 samples, lower frequency in three and equal in one; and this is a significant departure from random expectation ($\chi^2_1 = 5.88$, $P < 0.02$).

With six observed *IS*-sequences there are 21 possible different karyotypes for each sex. These are shown in table 2, from which inversion frequencies for males and females at Carlton Forehoe can be derived separately. Using total data the frequencies are (males/females): *IS-I.G*, 0.19/0.28; *IS-B*, 0.25/0.37; *IS-St*, 0.41/0.21; *IS-G*, 0.11/0.9; and *IS-I + B.G*, 0.04/0.05. The sex differences for *IS-I.G*, *IS-B* and *IS-St* can be tested by chi-square and are significant in each case at the 1 per cent level. Furthermore, in males, but not females, there is a general excess of heterozygote karyotypes compared with Hardy-Weinberg expectation (table 2: total males, homozygotes V. heterozygotes, $\chi^2_1 = 7.98$, $P < 0.01$; total females, $\chi^2_1 < 0.001$, $P > 0.99$). The excess of male heterozygotes, and sex

Table 3 Expected male *IL*-karyotype frequency distribution

Generation	Group	Karyotype			χ^2_2
		A/A	A/St	St/St	
1	Observed males	8	26	4	
	Observed females	2	25	35	
	Expected males ($P_A = 0.553$)	11.6	18.8	7.6	5.58
	Expected males ($P_{yA} = 0.871$, $P_{xA} = 0.234$)	7.7	26.6	3.7	0.05
2	Observed males	3	17	2	
	Observed females	2	10	21	
	Expected males ($P_A = 0.523$)	6.0	11.0	5.0	6.57
	Expected males ($P_{yA} = 0.833$, $P_{xA} = 0.212$)	3.9	15.2	2.9	0.70
3	Observed males	8	23	1	
	Observed females	2	20	21	
	Expected males ($P_A = 0.609$)	11.9	15.2	4.9	8.38
	Expected males ($P_{yA} = 0.940$, $P_{xA} = 0.279$)	8.4	22.2	1.4	0.16
5	Observed males	10	10	3	
	Observed females	0	5	7	
	Expected males ($P_A = 0.652$)	9.8	10.4	2.8	0.03
	Expected males ($P_{yA} = 1.000$, $P_{xA} = 0.208$)	4.9	18.1	0	11.93

Table 4 Expected male IS-karyotype frequency distribution

Generation	Group	I.G/I.G	I.G/B	I.G/St	I.G/Ot	B/B	Karyotype ¹				St/St	St/Ot	Ot/Ot	χ^2_9
							B/St	B/Ot						
All 1978	Observed males	3	12	24	5	2	35	10		13	15		4	
	Observed females	12	32	18	10	21	22	14		6	9		4	
	Expected males ²	4.5	11.7	19.1	7.3	7.6	24.8	9.4		20.3	15.5		2.9	13.91
	Expected males ³	3.4	8.8	23.7	7.6	5.7	30.9	9.9		15.4	14.7		2.9	5.85

1. Ot signifies sequences B,G, I & G together.
2. Assuming autosomal inheritance, $P_{I,G} = 0.1911$, $P_B = 0.2480$, $P_{st} = 0.4065$ and $P_{ot} = 0.1548$.
3. Assuming sex-chromosome linkage, $P_{st,G} = 0.0984$, $P_{st,G} = 0.2838$, $P_{st,B} = 0.1244$, $P_{st,B} = 0.3716$, $P_{st,st} = 0.2061$, $P_{st,st} = 0.1711$, and $P_{st,st} = 0.1385$.

difference in inversion frequencies suggest that the inversions occur at different frequencies on the X and Y chromosomes, and karyotype frequencies expected on this assumption give a closer fit to the observed data than autosomal Hardy-Weinberg expectations (table 4).

There were differences observed between "early" and "late" samples in the frequency of the six chromosome sequences in total females, but not in males (table 2, combining *IS-B.G* and *I*; males, $\chi^2_4 = 0.52$, $P = 0.97$; females, $\chi^2_4 = 13.22$, $P = 0.01$). Sequence *IS-B* is responsible for most of this variation, and this inversion shows an excess of *IS-B/B* homozygotes in total females from "late" samples compared with "early" samples (analysis not shown, but see table 2 for data).

In summary, the inversions of the *IS-B + I + G* system show linkage disequilibrium with each other and with the sex chromosomes, such that males are more frequently heterozygous than females. Furthermore, *IS-B* seems to be correlated with speed of larval development such that *IS-B/B* homozygote females, but not necessarily males, which are rare, develop more slowly.

DISCUSSION

Males of most species of blackfly, including *S. erythrocephalum*, are chiasmate with largely isomorphic sex chromosomes. This suggests that sex is primarily determined by a single locus, or at most a short length of chromosome. In *S. erythrocephalum* the primary sex chromosome differential region is the non-pairing region at the base of the short arm of the first chromosome, but this primary region can be enlarged by the serial linkage of inversions out along the chromosome. This pattern is quite common in blackflies (for references see Post, 1982a) and might be explained by the progressive linkage of new advantageous inversions to the sex locus. If such inversions include the sex locus, they will increase in frequency, but will be unable to cross over onto the other sex chromosome, even if they are advantageous in both sexes. Similarly, neutral inversions which happen to include the sex locus will be able to increase in frequency only on the sex chromosome on which they first appeared. Alternatively, the observed pattern of serial sex chromosome differentiation by inversion might be the result of selection for decreased recombination between X and Y. Charlesworth and Charlesworth (1980) have shown that there will be selection for decreased recombination between the sex locus and a second

selected locus if there is already a selectively maintained sex difference in allele frequency at the second locus. The existence of a series of such loci along the chromosome would result in the selection of inversions to progressively increase the size of the sex chromosome differential segment. This sort of selection might also explain the observed sex differences in the *IL-A* and *IS-B + I + G* inversion systems in *S. erythrocephalum*.

To give the observed karyotypes, *IL-A* and *IS-B + I + G* must be able to recombine with the sex chromosomes, although the rate of recombination is unknown. These inversions are polymorphic throughout southern England and *IS-B + I* (at least) have been found in populations around Leningrad (Petrushkina, 1968). Because they are widespread, these inversions are probably not new and hence the sex differences in frequencies should have had time to decay if they were not maintained by selection. The nature of this presumed selection is not clear but could be involved with the inversions' apparent effects on speed of larval development.

Males which emerge as adults earlier have more potential mates (Wiklund and Fagerstrom, 1977), which could give the *IS-A/St* males an advantage, because they are faster developing than the homozygotes. This male limited heterozygote advantage could maintain the *IL-A* polymorphism, but not the sex difference in frequency; for this there must also be selection on the female (Charlesworth and Charlesworth, 1980). Faster developing females are smaller and have fewer ovarioles (Ruhm and Hechler, 1974) but there is no evidence to suggest that *IL-A* is associated with speed of development in female *S. erythrocephalum*. However, *IS-B/B* females do develop more slowly and this is likely to be selected. To maintain the observed sex difference in frequency of *IS-B* there must also be selection in the males. It is possible that *IS-B/B* males also develop more slowly, but such male karyotypes are rare and it was not possible to test this hypothesis with the sample sizes obtained.

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