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Microsatellites reveal male recombination and neo-sex chromosome formation in *Scaptodrosophila hibisci* (Drosophilidae)

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Summary

In drosophilid flies, male recombination and neo-sex chromosome formation are rare. Following the genotyping of full-sib families with 20 microsatellite markers and subsequent cytological work, we found evidence of both male recombination and neo-sex chromosome formation in *Scaptodrosophila hibisci*. As far as we are aware, this is the first report of male recombination and neo-sex chromosome formation co-occurring in a drosophilid fly. Two autosomal loci, Sh29c and Sh90, showed aberrant segregation of male parental alleles. We describe how an autosomal fission followed by fusion of one of the autosomal fragments to the Y chromosome to create a $Y_1Y_2X_1X_2/X_1X_1X_2X_2$ sex determination system provides the most parsimonious explanation of the patterns we observe. Male recombination was observed in three families, including autosomal linkage groups and the Y_1/X_2 linkage group. In addition to the X_1 linkage group, two autosomal linkage groups were identified.

1. Introduction

In the Drosophilidae, both meiotic recombination in males and neo-sex chromosome formation are rare (Morgan, 1912, 1914; Ashburner, 1989). There is no evidence for, nor any clear *a priori* reason to expect, a connection between them. As part of a study of microsatellite variation in *Scaptodrosophila hibisci* (Barker, 2005), matings were set up to check for normal Mendelian segregation of loci. However, instead of Mendelian segregation, we found evidence of male recombination and neo-sex chromosome formation involving some of our 20 loci. We report those results here.

Spontaneous male recombination is known in a number of *Drosophila* species including *D. ananassae* (Kikkawa, 1937; Moriwaki, 1937), *D. bipectinata* (Singh & Banerjee, 1996), *D. melanogaster* (Hiraizumi, 1971), *D. simulans* (Woodruff & Bortolozzi, 1976), *D. virilis* (Kikkawa, 1933, 1935) and *D. willistoni* (Franca *et al.*, 1968), but in all these species except *D. ananassae*, recombination occurs at a very low rate (Ashburner, 1989). Male recombination falls mainly into two classes: (i) recombination that is meiotic in origin, such as occurs in *D. ananassae* (Matsuda *et al.*, 1983), and (ii) recombination associated with hybrid dysgenesis that is the result of *P* element activity, typically found in clusters and therefore traditionally thought to be pre-meiotic in origin (Kidwell *et al.*, 1977; Woodruff & Thompson, 1977).

Recent neo-sex chromosomal systems are known in three species of Drosophila: *D. miranda* (Dobzhansky, 1935), *D. americana* (Stalker, 1940, 1942; McAllister, 2002) and *D. albomicans* (Ranganath & Hagele, 1981). Such systems are useful natural models for studying the processes of sex chromosome evolution and in particular, Y chromosome degradation and dosage compensation (Steinemann, 1982; Steinemann & Steinemann, 1998; Charlesworth *et al.*, 2005). These three species represent phylogenetically independent examples of neo-sex chromosome

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systems and each provides a slightly different model system. The most well-characterized, *D. miranda*, is estimated to be about 1 million years old (Bachtrog & Charlesworth, 2000) and originated from the fusion of chromosome 3 to the Y chromosome, resulting in the creation of a neo-Y chromosome and thus $X_1X_1X_2X_2/X_1X_2Y$ sex determination (Dobzhansky, 1935). The neo-sex chromosomes of *D. americana* resulted from an X-4 fusion which generated XX/XY_1Y_2 sex determination (Stalker, 1940, 1942). This system is only a few hundred thousand years old (Throckmorton, 1982) and is not fixed, rather its frequency changes clinally from high values in the northern part of the species distribution to low values in the southern part (McAllister, 2002). Finally, the neo-sex chromosomes of *D. albomicans* are about 350 000–500 000 years old (Chang & Ayala, 1989) and resulted from two fusion events where chromosome 3 fused to both the X chromosome and also to the Y chromosome resulting in XX/XY sex determination (Ranganath & Hagele, 1981, Yu *et al*., 1999).

Scaptodrosophila hibisci is an endemic Australian member of the family Drosophilidae that breeds in the flowers of native *Hibiscus* species. Compared with the genus *Drosophila*, very little is known of the biology and genetics of *Scaptodrosophila* species. The results we present here identify *S. hibisci* as another model for the study of sex-chromosome evolution. Several theories on the origin of the Y chromosome attribute the degeneration of the Y chromosome to an absence of recombination (Charlesworth, 1996; Rice, 1996; but see Carvalho, 2002) and neo-Y chromosomes have been used to make tests of these theories (e.g. McAllister & Charlesworth, 1999; Steinemann & Steinemann, 2000; Bachtrog, 2004). As far as we are aware, this is the first report of both male recombination and neo-sex chromosomes occurring in a drosophilid fly and thus the *S. hibisci* system has potential to offer new insights into the role of recombination in Y chromosome evolution.

2. Materials and methods

(i) *Breeding and microsatellite screening of* Scaptodrosophila hibisci *families*

Thirty-two single pairs were established using males from a cage population derived from Bellingen, NSW, and both sexes from parental lines, F1 and F2 and backcrosses of two isofemale lines derived from single wild-caught females (Trd35 and Trd48) from Nimbin, NSW (Starmer *et al*., 2000). Each pair was supplied every day with a fresh glasshouse-grown *Hibiscus heterophyllus* flower, with the previous day's flower placed for immature-stage development in a 200 ml bottle containing dry, autoclaved sand. After pupae started to form, 10 ml of distilled water was added to each bottle to moisten the sand. Emerging adults were aspirated from the bottles each day, sexed and frozen at $-80\,^{\circ}C$ for later microsatellite assay. For nine of these 32 families, both parents survived to 12 days old (when they were frozen for later microsatellite assay) and produced reasonable numbers of progeny (mean = 38, range = 10–72).

Parental flies were genotyped at each of the 20 microsatellite loci described in Wilson *et al*. (2002). Sixteen of the loci were run as eight duplexes, and null individuals were identified by a complete absence of banding for one locus but normal bands for the other. For the remaining four loci, apparent null individuals were confirmed by repeat assay. Between five and 36 progeny of each sex per family were typed at informative loci, i.e. loci where the parental genotypes differed by at least one allele.

Chi-squared tests for deviations from Mendelian expectations were done for each locus in each family (Sokal & Rohlf, 1995).

(ii) *Linkage analysis*

Five of the loci ($Sh9^X$, $Sh72^X$, $Sh74^X$, $Sh78^X$ and $Sh89^X$) were shown previously to be X-linked by apparent hemizygosity of males (Wilson *et al*., 2002). Linkage groups of autosomal loci were determined from patterns of pairwise segregation of loci characterized within each family. When the male parent was heterozygous for both loci and two of the four possible male gametes in the progeny were absent, this was taken as evidence of linkage between the loci, under the assumption of little recombination in male drosophilids (references above).

(iii) *Segregation distortion*

Segregation ratios from the male parent were tested for deviation from the expected 1 : 1 ratio for each locus in each family. Evaluating these ratios for each pair of loci in each family allowed determination of the male parental genotype and haplotypic phase for each linkage group, normally equivalent to a chromosome (but see Section 4). Gametic segregation ratios for each chromosome were then estimated as a weighted average (because of differing numbers of progeny per locus) of the ratios for each locus. Segregation from the female parent was also evaluated, but female parental haplotypes could not be determined because of small family sizes.

(iv) *Cytology*

Flies used were from *S. hibisci* cultures maintained on *H. heterophyllus* or *H. diversifolius* flowers. Flowers were placed in glass or plastic bottles secured with a porous plug of foam or cotton wool, and larvae, pupae and adults were harvested as required by

Table 1. *Family studies to test Mendelian inheritance of loci*

Locus	Linkage group	Null allele segregating	No. of families	Total no. of progeny (χ^2 tests for deviation from Mendelian expectations)
Sh8i	2	+	7	240
Sh8ii	2	+	7	191
$Sh9^X$	X		4	69
Sh13	3		4	98
Sh29c	1	+	6	215 (see Table 2, $\chi^2_{(3)}=9{\cdot}5$* in family 4)
Sh34d	2	+	5	96
Sh36c	3	+	4	98 ($\chi^2_{(3)}=11{\cdot}14$* in family 1)
Sh38d	2	+	3	58
Sh48	Unassigned		1	20
Sh49	Unassigned	+	5	76
$Sh72^X$	X		4	58 ($\chi^2_{(1)}=5{\cdot}44$* for males in family 6)
$Sh74^X$	X		None	
$Sh78^X$	X		7	148 ($\chi^2_{(1)}=4{\cdot}0$* for females in family 9)
Sh81	3	+	4	62
Sh88	3	+	6	114
$Sh88^+$	2	+	5	92 ($\chi^2_{(1)}=11{\cdot}64$*** in family 1)
$Sh89^X$	X	+	6	94
Sh90	1	+	6	225 (see Table 2, $\chi^2_{(3)}=17{\cdot}5$*** in family 4, $\chi^2_{(3)}=21{\cdot}1$*** in family 9, $\chi^2_{(2)}=8{\cdot}4$* in family 17)
$Sh90^+$	Unassigned		2	46
Sh94	3	+	2	42

+, Null allele segregating; No. of families, number of families used to test Mendelian inheritance of the locus; None, no family segregating.
*$P<0{\cdot}05$, ***$P<0{\cdot}001$.

dissecting the flowers or removal from inside the bottles after larval exodus.

Thirty-six air-dried mitotic chromosome preparations were made from larval brains dissected in insect saline and processed using methods developed for the Australian sheep blowfly, *Lucilia cuprina* (Bedo, 1980). Thirteen of these slides were prepared from progeny of flies collected at Tyagarah, NSW and 23 from flies from Bellingen, NSW. Slides were stained in 4% Giemsa for initial examination and photography of well-spread metaphase cells. Twenty-nine air-dried meiotic preparations were made from pupal or adult testes using a similar procedure from descendants of flies collected at Tyagarah.

Slides were C banded using a 3 min saturated BaOH treatment followed by incubation in 2× SSC at 65 °C for 1 h (Bedo, 1980). Pre-photographed cells were then located and a second photograph taken for comparison. Photos were taken on Kodak Recordak film and developed in a 1:30 dilution of Agfa Rodinal. Photo negatives were scanned in a Polaroid Sprintscan 35LE film scanner and the resulting images processed in Adobe Photoshop.

3. Results

(i) *Confirmation of Mendelian inheritance of loci*

The loci generally conformed to Mendelian expectations (Table 1), but loci Sh29c and Sh90 were quite exceptional, with any given genotype generally found in only one sex of the progeny within a family (see Table 2). The patterns of inheritance for these two loci are considered in detail below. Of the other loci, two autosomal loci (Sh36c and $Sh88^+$) showed significant deviations from Mendelian expectations, but each only in family 1. Two of the X-linked loci ($Sh72^X$ and $Sh78^X$) also showed significant deviations, but only for one sex of offspring (Table 1). Following the sequential Bonferroni correction (Rice, 1989), only Sh90 in families 4 and 9 remained significant. However, as this procedure has reduced statistical power (Nakagawa, 2004), the probability of getting the observed eight (or more) significant tests out of the 88 performed was calculated using the likelihood function of Chapman *et al.* (1999) as 0·073. Thus, except possibly for Sh90 in two of the six families with data for this locus, all loci conform to Mendelian expectations.

(ii) *Neo-Y formation resulting from a Y-autosome fusion*

Although Sh29c and Sh90 appeared to be autosomal, having clear heterozygous males, both show linkage to the X-linked loci and aberrant segregation of male parental alleles. For Sh29c, male gametes carrying the *109* allele transmit only to male progeny (the exceptions in family 1 are discussed below, see section

Table 2. *Family data for loci Sh29c and Sh90*

	Sh29c					Sh90				
Family	Father	Mother	Progeny	Male progeny	Female progeny	Father	Mother	Progeny	Male progeny	Female progeny
1	*109/null*	*121/null*	*109/121*	13	4[a]	*null/136*	*138/null*	*null/138*	14	0
			109/null	16	3[a]			*null/null*	13	0
			null/121	0	12			*136/138*	0	15
			null/null	0	12			*136/null*	0	14
4	*109/121*	*109/121*	*109/109*	1	0	*138/136*	*136/138*	*138/136*[b]	3	0
			109/121[b]	4	0			*138/138*	2	0
			121/109[b]	0	2			*136/136*	0	0
			121/121	0	9			*136/138*[b]	0	11
6	*109/108*	*121/121*	*109/121*	10	0	*138/137*	*136/138*	*138/136*	7	0
			108/121	0	14			*138/138*	12	0
								137/136	0	15
								137/138	0	9
9	*109/121*	*109/121*	*109/109*	7	0	*138/137*	*137/138*	*138/137*[b]	5	0
			109/121[b]	5	0			*138/138*	7	0
			121/109[b]	0	7			*137/137*	0	0
			121/121	0	10			*137/138*[b]	0	17
17	*109/121*	*108/null*	*109/108*	16	0	*138/136*	*137/138*	*138/137*	20	0
			109/null	11	0			*138/138*	7	0
			121/108	0	10			*136/137*	0	11
			l21/null	0	10			*136/138*	0	9
20	*109/121*	*109/121*	*109/109*	10	0	*138/137*	*136/138*	*138/136*	7	0
			109/121[b]	10	0			*138/138*	8	0
			121/109[b]	0	7			*137/136*	0	14
			121/121	0	12			*137/138*	0	5

[a] Progeny from male recombination between Sh29c and Sh90.
[b] Within a family, these are equivalent genotypes. Consistent with allele segregation in other families at these loci, we have assigned male and female progeny to these cells. Alternative inheritance is possible but only if male recombination is invoked.

3(iv)), while those carrying *108*, *121* or *null* alleles transmit only to female progeny. Similarly for Sh90, male gametes carrying the *138* or *null* alleles transmit only to male progeny, while those carrying *136* or *137* alleles transmit only to female progeny (Table 2). The male parents of families 1, 4 and 17 had their Y chromosome from the Trd 48 isofemale line, while the male parents of families 6, 9 and 20 came from the Bellingen cage population. Thus the aberrant segregation of male parental alleles was not population-specific, and the results suggest a Y−autosome fusion.

(iii) *Three linkage groups identified*

Analysis of the family data indicated three linkage groups: one X-linked (group 1, Wilson *et al.*, 2002) and two autosomal (Table 1). All pairwise combinations of five loci (Sh8i, Sh8ii, Sh34d, Sh38d, Sh88$^+$) showed linkage, as male recombination was not observed in any of the one to four families per pair. Similarly, Sh13, Sh36c, Sh81 and Sh94 comprise a second autosomal linkage group, with no male recombination observed in one to two families per locus pair. Sh88 is tentatively assigned to this linkage group on the basis of one apparent recombinant in 10 progeny from the joint segregation of Sh81 and Sh88 from the male parent in one family. The female parent of this family was homozygous at Sh88, so no supporting data are available from female recombination frequencies. The aberrant loci (Sh29c and Sh90) are linked, as no male recombinants were observed in their joint segregation in four families. However, these two loci are part of linkage group 1, as male recombinants were absent in the joint segregation of Sh29c with each of Sh72^X and Sh78^X, and of Sh90 with Sh72^X, Sh78^X and Sh89^X.

Although family sizes are small, results for male recombination (see below), together with observed female recombination frequencies, allow inference of the order of the loci in each autosomal linkage group (LG). In the autosomal linkage groups (2 and 3), loci are listed in order: LG 2 – Sh34d, Sh8i, Sh8ii, Sh38d and Sh88$^+$; LG 3 – Sh13, Sh36c and Sh88. Loci Sh81 and Sh94 also belong to LG 3 but it was not possible to assign order to these loci. Loci Sh48, Sh49 and Sh90$^+$ could not be assigned.

(iv) *Male recombination*

In five cases, locus pairs that are inferred to be linked gave progeny that could be accounted for only as a result of meiotic recombination in the male parent. These were (with number of recombinant progeny/total progeny): family 1, between loci Sh8i and Sh38d in 1/20 progeny (and also between Sh8ii and Sh38d) (LG 2), and between loci Sh29c and Sh90 in 7/60 progeny (sex-linked LG1); family 4, between Sh8ii and $Sh88^+$ in 4/9 progeny (LG 2), and between Sh81 and Sh88 in 1/10 progeny (LG 3); family 6 between each of Sh8i, Sh8ii and Sh38d with Sh34d (LG 2) in 8/20 progeny. That is, there were three cases of male recombination for LG 2, one for LG 3 and one for the aberrant loci Sh29c and Sh90.

(v) *Segregation distortion*

Male segregation ratios significantly different from 1 : 1 for individual loci were detected for both linkage groups 2 and 3 in families 1, 4 and 28, with the proportion of the more frequent gamete in the progeny averaging (over the three families) 0·696 for LG 2 and 0·771 for LG 3. No significant male segregation distortions were found for Sh29c and Sh90.

Segregation distortion from the female parent also was evaluated over all loci and families. Excluding Sh29c and Sh90, 10 segregation ratios for individual loci (out of 63 tests) were significantly different from 1 : 1. However, the tests are not independent for linked loci, and most significant tests could be ascribed to reduced viability of one progeny genotype. Further detailed family studies are needed to determine the generality of these apparently reduced viabilities.

For Sh29c and Sh90, female segregation ratios were significantly different from 1 : 1 in three families. Progeny numbers are given in Table 2, with both Sh29c and Sh90 significant in family 4 (ratio 3 : 13, $\chi^2 = 6{\cdot}25$, $P < 0{\cdot}05$), and Sh90 in family 9 (ratio 5 : 24, $\chi^2 = 12{\cdot}45$, $P < 0{\cdot}001$) and family 17 (ratio 31 : 16, $\chi^2 = 4{\cdot}79$, $P < 0{\cdot}05$). Note that these are the same locus/family combinations that showed significant deviations from Mendelian expectations (Table 1). For families 4 and 9, the patterns of deviation from expectation are the same, with one female genotype missing. Joint analysis of Sh29c and Sh90 (Table 3) shows the segregation to be more complex, with two genotypes missing in females of families 4 and 9, and other apparent distortions.

(vi) *Cytology: mitotic chromosomes*

Mitotic chromosome preparations clearly show that *S. hibisci* has six chromosome pairs (2n = 12, example in Fig. 1; cells from 11 individuals from Bellingen were photographed and 5 from Tyagarah, and additional ones not photographed reinforced the patterns). The karyotype is characterized by a large metacentric chromosome pair (chromosome 1), four intermediate chromosome pairs that form a graded series of three metacentric pairs (chromosomes 2–4) and a smaller acrocentric pair (chromosome 5), and a pair of small dot chromosomes (chromosome 6, Fig. 1). In the nomenclature used for *Drosophila* karyology (Clayton & Wheeler, 1975), the *S. hibisci* karyotype is 4V 1J 1D. This configuration is unique among the karyotypes of *Scaptodrosophila* species reported by Clayton & Wheeler (1975), indicating a high degree of heterogeneity in karyotypes of this genus.

Close inspection of mitotic chromosomes from a number of individuals shows that there is variation in the staining and size of some chromosome homologues. This is pronounced in chromosome 1 where we identified up to four distinct homologue forms (Fig. 2). These chromosome 1 homologues are found in two different combinations representing 'type I' and 'type II' cells. In type I cells, one chromosome 1 homologue has a lightly staining long arm showing chromatid separation. The short arm is mostly dark-staining with a secondary constriction near the end of the arm and less intense staining distal to the constriction. The other chromosome 1 homologue in type I cells is a shorter, intermediately stained structure with some dark bands and no chromatid separation. Type II cells have two differently banded chromosome 1 homologues: the difference appears to be the result of a large pericentric inversion. Both homologues in type II cells have pale-staining segments at the distal end of the long arm, showing chromatid separation.

Type I and type II cells were observed in mitotic chromosome preparations from two populations of *S. hibisci*. In the Bellingen sample, five individual flies gave rise to clear spreads: three of type I karyotype and two type II. In the Tyagarah material, two individuals yielded high-quality spreads, one of each type. Slides were prepared from larvae, for which a successful morphology-based sexing method was not developed here. Although numbers are small in each population, type I and type II karyotypes were found in a 1 : 1 ratio, raising the possibility that these two karyotypes are associated with gender. Future work will be required to test this hypothesis.

(vii) *Cytology: meiotic chromosomes in males*

It was not possible to separate meiotic metaphase chromosomes adequately for detailed analysis of the sex chromosome constitution. However, of the meiotic preparations from 29 flies, three slides provided clearly visible meiotic spreads. In these preparations, chromosomes are visibly linked into several tightly

Table 3. *Bi-locus genotypes of progeny at loci Sh29c and Sh90*

Maternal gametes		Paternal gametes			
		Parental		Recombinant	
	FAMILY 1	*109, null*	*null, 136*	*109, 136*	*null, null*
Parental	*121, 138*	5 m	7 f	1 f	0
	null, null	6 m	6 f	1 f	0
Recombinant	*121, null*	4 m	5 f	3 f	0
	null, 138	4 m	4 f	2 f	0
	FAMILY 4	*109, 138*	*121, 136*	*109, 136*	*121, 138*
Parental	*109, 136*	0	0	0	D
	121, 138	1 m	9 f	D	0
Recombinant	*109, 138*	1 m	2 f	D	D
	121, 136	3 m	0	D	D
	FAMILY 6	*109, 138*	*108, 137*	*109, 137*	*108, 138*
Parental	*121, 136*	1 m	8 f	0	0
	121, 138	9 m	6 f	0	0
	FAMILY 9	*109, 138*	*121, 137*	*109, 137*	*121, 138*
Parental	*109, 137*	5 m	0	0	D
	121, 138	5 m	10 f	D	0
Recombinant	*109, 138*	2 m	7 f	D	D
	121, 137	0	0	D	D
	FAMILY 17	*109, 138*	*121, 136*	*109, 136*	*121, 138*
Parental	*108, 138*	6 m	9 f	0	0
	null, 137	10 m	10 f	0	0
Recombinant	*108, 137*	10 m	1 f	0	0
	null, 138	1 m	0	0	0
	FAMILY 20	*109, 138*	*121, 137*	*109, 137*	*121, 138*
Parental	*109, 136*	6 m	3 f	0	0
	121, 138	7 m	1 f	0	0
Recombinant	*109, 138*	1 m	4 f	0	0
	121, 136	1 m	10 f	0	0

Alleles for locus Sh29c appear before alleles for locus Sh90, e.g. *109*/*null*: allele *109* at locus Sh29c and *null* allele at locus Sh90. Male parental gametes are listed on the horizontal for each family and female parental gametes on the vertical. Maternal gametes are defined as Parental or Recombinant on the basis of relative frequency, i.e. Parental as the more frequent. Male and female progeny of each genotype are indicated by m and f respectively, and 0 indicates no progeny of that genotype. Note for families 4 and 9 there are only 10 different possible progeny genotypes and that for five of them there are two possible ways to arrive at that genotype depending upon which parent each haplotype is inherited from, and for one there are three possible ways. We have assigned progeny in this table conservatively, i.e. as if there is no male recombination and only two paternal gamete types. D, duplicate genotype.

paired chains with dark-staining segments distributed through the chains, indicating involvement of multiple chromosomes (Fig. 3). Four such complexes are shown in Fig. 3. A large darkly stained region in one of the chromosome groups suggests that this involves the first chromosome pair. The other smaller darkly stained areas in the remaining three chains probably correspond to the heavily stained regions in chromosomes 2 to 5. Because of its small size, chromosome 6 could not be identified in the complex pairing structures.

4. Discussion

(i) *A rare example of male recombination and neo-sex chromosome formation in the same species*

This is the first report, as far as we are aware, of both male recombination and neo-sex chromosome formation co-occurring in a drosophilid fly. One study in the Mediterranean fruit fly, *Ceratitis capitata*, has shown that whilst male recombination is rare, the occurrence of a Y−autosome translocation is associated with low but observable rates of recombination in males carrying the rearrangement (Rössler, 1982). A similar pattern was reported in the Australian sheep blowfly, *Lucilia cuprina* (Foster *et al*., 1980). However, in a subsequent study it was shown that the structural integrity of the Y chromosome had little or no influence on crossing-over in male *L. cuprina* (Foster *et al*., 1991). In the present study we observed male meiotic recombination in three families, affecting two autosomal linkage groups and the putative neo-Y_1/X_2 linkage group. Recombination frequencies were very variable, ranging from 0·05 to 0·44, but as there were no useful data from females, these frequencies cannot be related to linkage

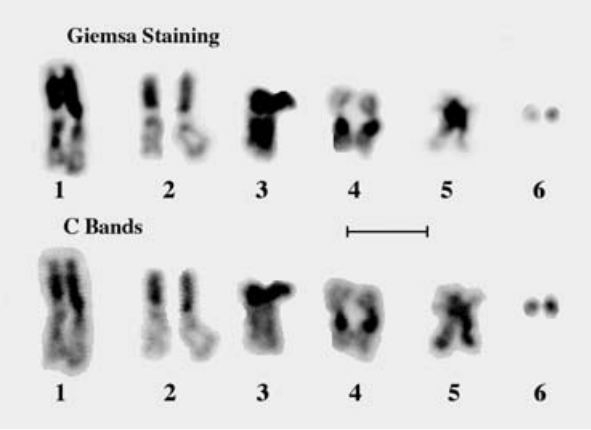

Fig. 1. Giemsa and C banded karyotypes of a type II cell arranged in descending order of size. Heterochromatic areas are shown by dark Giemsa and C banding in all chromosomes. The long arms of chromosomes 4 and 5 and chromosome 6 have differential staining with Giemsa and C bands. There is also differential staining between chromosome homologues, particularly in chromosome 1 and chromosome 6. Scale bar represents 5 μm.

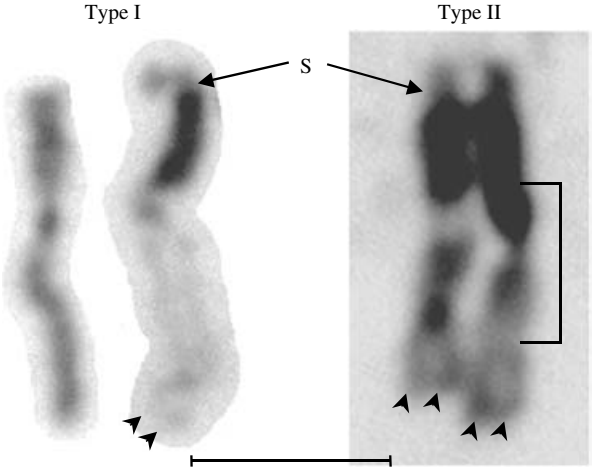

Fig. 2. Enlargement of Giemsa-stained chromosome 1 homologues from type I and II cells showing heterozygosity of banding patterns. A region of rearranged banding in one chromosome in the type II cells, probably involving a pericentric inversion, is delimited by a bracket. Secondary constrictions (S) and distal satellites are visible in the short arm of three chromosomes. Separate chromatids can also be resolved in the long arms of these chromosomes (arrowheads). Scale bar represents 5 μm.

distances. Since male recombination was observed for each of linkage groups 2, 3 and loci Sh29c/Sh90, there appears no obvious mechanism that would relate it to the neo-sex chromosome system. However, the complex karyotypic rearrangements we observed in *S. hibisci* may have the effect of facilitating crossing-over in males that normally are achiasmate. In fact, the very close pairing seen in meiotic preparations (Fig. 3) is suggestive of chiasmate meiosis and crossing-over. Further investigation of meiosis in *S. hibisci* should include synaptonemal complex formation.

(ii) *Models of sex chromosome rearrangement in* S. hibisci

In analysing the data we present in this paper we have painstakingly considered all possible models of sex chromosome evolution to explain the aberrant segregation of alleles in *S. hibisci* males at microsatellite loci Sh29c and Sh90. The models we have considered include the presence of a sex chromosome pseudoautosomal region as well as fusion of all or part of an autosome to the ancestral X or Y chromosomes. Sex chromosomes evolved in the flies prior to divergence of *Scaptodrosophila* and *Drosophila* (Saccone *et al.*, 2002); hence, ancestral sex determination in *S. hibisci* was with high probability of the XX/XY type.

Pseudoautosomal regions of sex chromosomes are regions of homology between X and Y chromosomes and sites of recombination between often highly differentiated chromosomes. If loci Sh29c and Sh90 were associated with a sex chromosome pseudoautosomal region, we would expect to observe all Sh29c and Sh90 alleles distributed, as a result of recombination, on the X-linked and Y-linked pseudoautosomal regions. This is not what we found. We found only a small subset of alleles transmitted from male parents only to male progeny. Therefore, because: (i) XY sex determination predates the divergence of *Drosophila* and *Scaptodrosphila* (Saccone *et al.*, 2002) and (ii) a restricted set of Sh29c and Sh90 alleles are transmitted from fathers only to sons, a sex chromosome pseudoautosomal model does not adequately account for the patterns of inheritance we report here.

Any model involving fusion of a whole autosome to the ancestral X or Y chromosomes and some models, prior to fixation, involving fission of an autosome followed by fusion of one fragment to the X or Y chromosome, are not supported by our cytological work. We have established that both sexes of *S. hibisci* have a chromosome complement of $2n = 12$. Thus, all models that result in sex differences in the number of chromosomes can be eliminated from further consideration.

Models involving an X–autosome (X–A) translocation will result in unbalanced zygotes, and unless the translocation has become fixed, will result also in sex-limited transmission of Sh29c and Sh90 alleles in only some males. We observed sex-limited transmission of alleles at loci Sh29c and Sh90 in all males from two populations. Whilst there are systems with complex sex chromosome rearrangements involving translocation of parts of autosomes to X chromosomes (Rowell, 1985; John, 1987; Luykx, 1990) and X–A translocations have been shown to be

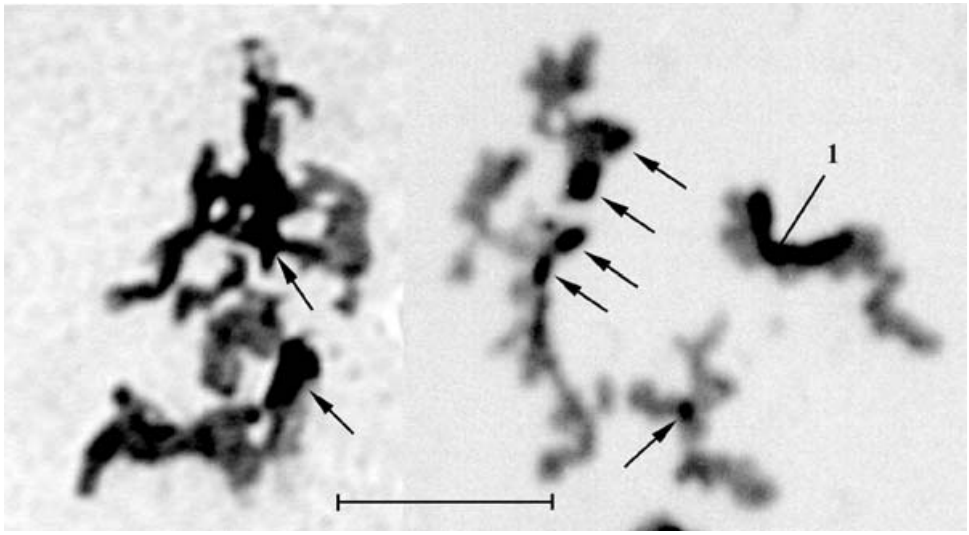

Fig. 3. Meioses showing tight pairing in several putative translocation chains. A large heterochromatic region, possibly from chromosome 1, can be identified in one of the chains (labelled 1). Smaller darkly stained heterochromatic regions (arrows) are scattered through the other chains indicating involvement of multiple chromosomes. Scale bar represents 5 μm.

evolutionarily stable (Charlesworth & Wall, 1999), X−A rearrangements are less commonly observed than Y−A rearrangements and are frequently maintained as polymorphisms within a species (e.g. Luykx, 1990; McAllister, 2002). Because we find no evidence of chromosomal polymorphism within *S. hibisci* and because all males exhibited sex-limited transmission of Sh29c and Sh90 alleles, we argue that a system involving an X−A translocation is less parsimonious than one involving reciprocal translocation of part of an autosome to the ancestral Y chromosome. However, further work is required to fully eliminate the possibility that an X−A translocation accounts for our data.

In considering possible models of sex chromosome evolution, we favour a model most similar to that of *D. miranda* (and see discussion of cytological work below) (Dobzhansky, 1935). Subsequent to the divergence of *Drosophila* from *Scaptodrosophila*, probably at some time in the recent past, there has been a chromosomal rearrangement where part of an autosome bearing loci Sh29c and Sh90 was reciprocally translocated with part of the ancestral Y chromosome to form a X_1X_1 X_2X_2/X_1X_2 Y_1Y_2 sex determination system (Fig. 4).

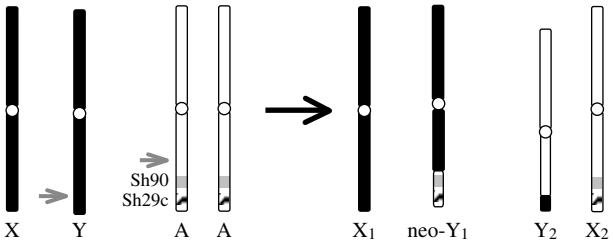

Fig. 4. Proposed scheme for neo-sex chromosome rearrangement in *S. hibisci*, with translocation of part of an autosomal arm bearing loci Sh90 and Sh29c to the Y chromosome. We assume isomorphic sex chromosomes, fission in the original Y, and a reciprocal translocation, although our evidence does not exclude the translocated part of the autosome having fused to the end of the Y. Small grey arrows indicate chromosome break points. Solid grey band includes locus Sh90, hatched band includes locus Sh29c.

(iii) *Cytology*

The heterozygosity of the *S. hibisci* karyotype and likely translocation chains with no independent bivalents in meiotic metaphase suggests this species has a complex sex chromosome system involving autosomal fragmentation and rearrangements between autosomes and ancestral X and Y chromosomes. Although adhesions may result in failure to separate meiotic chromosomes during slide preparation, if a technical artefact were the main cause of the observations, one would expect clumping to be less tenacious and individual bivalents to be separated in some preparations. Chromosomes are often seen to be associating end-to-end, again more consistent with complex rearrangements than sticky chromosomes. Also the heterozygosity of banding and chromosome size, in particular of chromosome 1, suggest translocation complexes are present. *Drosophila americana americana*, *D. miranda* and *D. albomicans* have respectively neo-X, neo-Y or both neo-X and neo-Y chromosomes formed by translocation between the X, Y or both and an autosome, but the degree of heterozygosity and complex pairing suggest that *S. hibisci* has additional rearrangements. This conclusion is consistent with the microsatellite data we present here; however, microsatellite genotyping

indicated only a single autosome–sex chromosome translocation.

The two cell types defined by chromosome 1 homologue forms most probably represent male and female individuals. Unfortunately, unsuccessful attempts to identify larval sex during slide preparation, and inability to identify chromosome 1 homologues in testis preparations leaves this question open. In future we recommend scoring loci Sh29c and Sh90 in the remains of larvae used for cytology: given parental genotypes, sex of the offspring could then be estimated. However, the intermediately stained chromosome seen in some individuals may represent a partially heterochromatinized neo-Y chromosome as proposed in the degeneration model of Y chromosome evolution (Steinemann, 1982; Steinemann & Steinemann, 1998). If this is the case, *S. hibisci* offers opportunities for study of Y chromosome evolution in a highly differentiated sex chromosome system.

Although there are six chromosome pairs in the mitotic karyotype of *S. hibisci*, only three linkage groups were found in the study of microsatellite inheritance. The suggested presence of translocation rearrangements in *S. hibisci* would result in fewer than six linkage groups. However, stochastic locations of 20 microsatellites on six chromosomes of greatly differing sizes could also contribute to the shortfall in number of linkage groups.

The cytology of *S. hibisci*, particularly extensive karyotype rearrangement resulting in homologue heterozygosity and possible translocation chains in meiosis, is consistent with conclusions from the genetic marker family studies: both support the existence of a complex sex chromosome system involving neo-X and neo-Y chromosomes. The sex chromosome system of *S. hibisci* appears to be exceptional among drosophilids.

(iv) *Segregation distortion and progeny inviabilities*

The joint segregation of Sh29c and Sh90 (Table 3) shows several empty cells where one would expect to observe progeny. Because of the small family sizes, we comment on only three classes of missing progeny. In family 1, only three of four possible paternal gametes result in viable progeny: (i) male progeny that inherit a non-recombined neo-Y from their father ($Sh29c^{109}$, $Sh90^{null}$), (ii) female progeny that inherit a non-recombined X_2 from their father ($Sh29c^{null}$, $Sh90^{136}$) and (iii) female progeny that inherit a recombined X_2 from their father ($Sh29c^{109}$, $Sh90^{136}$). The segregation of these females indicates male recombination between Sh29c and Sh90, and that Sh90 is closer than Sh29c to the end of X_2 that is fused to the Y. Male gametes that carry a putative recombined neo-Y (Y, $Sh90^{null}$, $Sh29c^{null}$) are apparently too rarely formed to be detected here, or are associated with inviability.

The other two examples of missing progeny genotypes (families 4 and 9) have in common that the missing genotypes are homozygous for the Sh90 allele carried by the mother, or looked at another way, homozygous for the neo-X chromosome Sh90 allele from the male parent. We currently have no explanation for these apparent inviabilities, but they may be illuminated by further karyotyping and genetic analysis.

(v) *Conclusions and future directions*

The finding of both neo-sex chromosomes and male recombination in *S. hibisci* was quite unexpected. Yet this is the first *Scaptodrosophila* species whose genetics has been studied in this way; thus these phenomena are not necessarily rare in the genus *Scaptodrosophila*, as they are in the genus *Drosophila*. As the *Scaptodrosophila* radiation is the oldest within the Drosophilidae (Throckmorton, 1975), male recombination may be the primitive condition retained to an extent in *S. hibisci*; equally, perhaps the presence of substantial chromosome rearrangements in the present species promotes male recombination. Among the species of the Drosophilidae, there is an enormous range of metaphase chromosome configurations (Clayton & Wheeler, 1975), i. e. many modifications of the primitive karyotype have evolved. Thus finding a neo-Y chromosome system in *S. hibisci* more likely is fortuitous, but similar genetic studies of other *Scaptodrosophila* species may well contribute to a substantial increase in our understanding of the evolution of sex chromosomes.

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