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Fusions of Muller's Elements during Chromosome Evolution of *Drosophila albomicans*

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Te-Pin Chang, Tai-Hua Tsai, and Hwei-yu Chang (2008) Fusions of Muller's elements during chromosome evolution of *Drosophila albomicans*. *Zoological Studies* **47**(5): 574-584. The karyotype of *Drosophila albomicans* differs from that of its sibling species *D. nasuta* by 2 fusions between a pair of autosomes and the sex chromosomes. All species of the *D. nasuta* subgroup exhibit the fusion events involved in the basic karyotype of the *D. immigrans* species group, but 2 additional fusions evolved in *D. albomicans*. This sibling species pair serves as a useful tool aided by the abundant DNA sequence information for *D. melanogaster* to correlate their chromosome arms to Muller's elements. Since the homologue of the 3rd autosome of *D. nasuta* is the long arm of the neo-sex chromosome of *D. albomicans*, genetic markers on it can be determined by checking sex-linkages from *D. albomicans* and *D. nasuta* hybrid crosses. Three genetic markers were found to be located on this chromosome arm by crossing experiments and by female homozygotes and male heterozygotes in certain isofemale strains. They were identified by BLAST as homologous to loci on the 2R and 3L chromosome arms of *D. melanogaster*, and therefore indicate correspondence of this arm to Muller' s elements C and D. After a survey of sexual differences with several chosen sequences, 11 more loci were found which were also located on Muller's elements C and D with no exceptions. By *in situ* hybridization, the homologies were confirmed: 2L = B, 2R = E, and 3 = C + D. Based on the hypothesized chromosome arm homologies between *D. melanogaster* and *D. albomicans*, the fusion events in lineages of *Sophophora* and *Drosophila* are discussed. http://zoolstud.sinica.edu.tw/Journals/47.5/574.pdf

Key words: *Drosophila nasuta*, Homoplasy, Karyotype, Neo-sex chromosome.

The subgenera *Sophophora* and *Drosophila* of the genus *Drosophila* diverged about 40 million yr ago (Russo et al. 1995); the former includes the well-studied model species *D. melanogaster*, and the latter contains a very interesting species, *D. albomicans*, which has recently evolved sex chromosome arms representing 40% of the genome (Chang and Ayala 1989, Yu et al. 1999). *Drosophila albomicans* with this young and huge neo-sex chromosome is an excellent species for testing hypotheses on sex chromosome evolution. Our previous studies reported the dependence of a neo-Y chromosome on the conspecific neo-X chromosome (Lin et al. 2007). Stronger male-male competition and female choice in *D. albomicans* compared to its sibling species, *D. nasuta* (Chang and Tai 2007), may also be related to its neo-sex chromosome arm. Comparisons of DNA sequences on sex vs. non-sex chromosomes will be of great value. *Drosophila albomicans* and *D. nasuta* belong to the *D. nasuta* species subgroup of the *D. immigrans* species group. Since *D. albomicans* is a non-model species and hence lacks basic genetic information, it is difficult to study chromosome evolution at the gene level. If homologies of chromosome arms between *D. albomicans* and *D. melanogaster* could be identified, the wealth of annotated sequence information from *D. melanogaster* could be inferred.

To alleviate the confusion caused by

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inconsistent terms for chromosome arms used in different species, we adopted "Muller's elements" to refer to the chromosome arm homology among *Drosophila* species (Muller 1940, Sturtevant and Novitski, 1941). They are denoted from A through F to respectively correspond to the X, 2L, 2R, 3L, 3R, and 4th chromosome arms of *D. melanogaster*. Karyotype relationships of several *Drosophila* species referenced to Muller's elements were reviewed in Ashburner et al. (2005). Neither *D. albomicans* nor any other species of the *D. immigrans* group was on that list. Meera Rao and Ranganath (1991) proposed a chromosome evolution scenario for the *D. immigrans* species group by analyzing karyotypes of 34 taxa belonging to 5 subgroups. Accordingly, multiple fusion events and a pericentric inversion were found to have been involved in chromosome evolution from 2*n* = 12 to 2*n* = 6. In this species group, 2 fusions evolved in the ancestral karyotype (2*n* = 12) to the basic one (2*n* = 8). Within the *D. immigrans* species group, species of the *D. nasuta* subgroup, except for *D. albomicans*, possess the basic karyotype (1m + 2t + 1dot) (Wakahama et al. 1983). Still, the chromosome relationships between *D. albomicans* and *D. melanogaster* are unknown.

The crossability (i.e., producing viable and fertile hybrids) between *D. albomicans* and *D. nasuta* (Kitagawa et al. 1982, Chang and Ayala 1989) provides an opportunity to discriminate loci on the neo-sex chromosome arm versus those on autosomes. By interspecific hybridization and backcrossing, one can determine whether a gene is located on this sex-linked chromosome arm. For a recessive morphological marker, counting thousands of hybrid flies for each cross is unavoidable (Ashadevi et al. 2005). Although the sample size can be largely reduced if codominant markers are adopted, it is hard to find codominant morphological markers in either *D. albomicans* or *D. nasuta*. Moreover, it is difficult to find homologous morphological markers between such distantly related species as *D. albomicans* and *D. melanogaster*. In this study, we attempted to establish molecular markers which can be used to correlate chromosome arms between different species.

It is convenient to use molecular markers with restriction site differences. In order to find polymerase chain reaction (PCR) restriction markers, it is appropriate to construct complementary (c)DNA libraries. Homologous sequences with interspecific differences can be used as genetic markers for crossing experiments to find those on the 3rd chromosome arm of the neo-sex chromosome. Afterwards, homologues of these molecular markers can be found in *D. melanogaster* with the aid of the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). A hypothesis of the Muller's element composition in *D. albomicans* was proposed based on the BLAST result. More loci were found by a restriction survey of sexual differences to test the robustness of this hypothesis. Furthermore, *in situ* hybridization to polytene chromosomes was used to verify the hypothesis. With the homologies indicated by Muller's elements, karyotypic fusion events during the evolutionary pathway of *Sophophora* and *Drosophila* were addressed.

MATERIALS AND METHODS

Fly strains

Four isofemale strains were used in this study. Two strains of *Drosophila albomicans*, #163.5 and #296.6, were respectively collected from Okinawa, Japan and Taiwan. Both *D. nasuta* strains #193.7 and #252.11 originated from India. Flies were reared and treated with a previously described method (Yu et al. 1999, Chang and Tai 2007). The *D. albomicans* #296.6 strain was used for the PCR restriction survey of sexual differences.

Crossing scheme

Two sets of crosses were performed with a *D. albomicans* female and a *D. nasuta* male. One was a #296.6 ♀ × a #252.11 ♂, and the other was a #163.5 ♀ × a #193.7 ♂. F_1 males were then backcrossed to *D. albomicans* females of the same genotype as the G_0 mother. If a genetic marker is located on an autosome such as the 2nd chromosome, either sex in the F_2 generation will have both homozygotes and heterozygotes. If the marker is located on the 3rd chromosome arm of the neo-sex chromosome of *D. albomicans*, all F_2 males will be heterozygous and all females homozygous (Fig. 1).

Strategies for finding molecular markers

Restriction fragment length polymorphism (RFLP) markers are ideal to serve as genetic

markers for genotyping but sequences are needed to begin. We established *cDNA libraries* of both species. Based on the expressed sequence tags (ESTs), we found candidate sequences as genetic markers for the crossing experiment, indicators for the survey of sexual differences, and probes for *in situ* hybridization.

cDNA libraries and ESTs. About 10 g of *D. albomicans* or *D. nasuta* flies aged 4-7 d were harvested for extraction for total RNA with TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Further purification of messenger (m)RNA was carried out with an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany), and about 10 μg mRNA was obtained. cDNA was synthesized from the mRNA using the Creator SMART cDNA Library Construction Kit (BD Clontech, Mountain View, CA, USA) following the manufacturer's protocol, and then digested with the *Sfi*I restriction enzyme before being ligated into the pDNR-LIB cloning vector (BD Clontech) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The ligation mixture was directly transformed into DH10B-competent cells (Epicentre Technologies, Madison, WI, USA) by electroporation. Transformed bacteria were grown on LB agar plates containing chloramphenicol (12.5 μg/ml). After the addition of X-gal and IPTG (isopropyl-beta-D-thiogalactoside) for blue/white screening, the corrected clones were identified as white colonies. Plasmid DNA was obtained from individual clone cultures using the QIAprep Spin Miniprep Kit (Qiagen). DNA sequencing was performed using this plasmid DNA as a template, with standard T7 as the 5' primer, and ABI BigDye chemistry vers. 3 (Perkin-Elmer, Waltham, MA, USA) on an ABI377 automated sequencer.

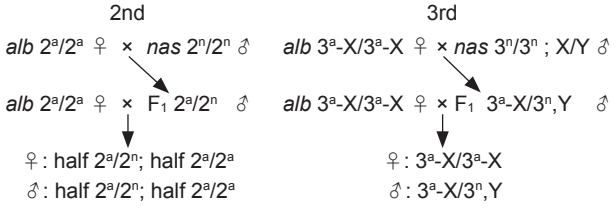

Fig. 1. Crossing scheme used to determine whether a gene is located on the 2nd chromosome or on the 3rd chromosome arm of the neo-sex chromosome of *Drosophila albomicans*. The superscript indicates what species the chromosome belongs to, e.g., 2^a or 2^n. An F_1 male produced by a cross between a *D. albomicans* (*alb*) female and a *D. nasuta* (*nas*) male was backcrossed to a *D. albomicans* female. If the gene is located on the 2nd chromosome in F_2, either sex has both homozygotes and heterozygotes. On the contrary, if it is located on the 3rd arm of the neo-sex chromosome of *D. albomicans*, all F_2 males are heterozygous, and all females are homozygous.

Markers for crosses. Sequences obtained from the cDNA libraries were assembled by the subroutine SeqMan of the program DNA* (DNASTAR, Madison, WI, USA) to find homologous sequences between *D. albomicans* and *D. nasuta*. Interspecific restriction differences were determined using the program MacVector 8.0 (Accelrys, San Diego, CA, USA). After primers were designed using the GCG (Genetics Computer Group) (Womble 2000) and MacVector 8.0, these PCR sequences served as genetic markers for interspecific hybridization and sex linkage determinations.

Markers for sexual differences. In addition to sequences shared by both species, we focused on *D. albomicans* ESTs to conduct restriction surveys. Sequence similarity searches were performed using the BLAST method (Altschul et al. 1990). Several criteria were established: a good quality length exceeded 500 bp; BLAST was used to examine *D. melanogaster* genes located on the 2nd or 3rd chromosomes; e-values had to be < 10^{-20}; and there had to be > 75% match. A published sequence of *D. albomicans*, *Amyrel*, obtained from NCBI (accession no.: AF462595, submitted by Da Lage et al.) was also used for primer design.

Genotyping

Single fly genomic DNA extraction. Genomic DNA of a single fly was extracted by means of the PUREGENE DNA Extraction Kit (Gentra Systems, Minneapolis, MN, USA). Each fly was homogenized in 100 μl of a Cell Lysis Solution. After the RNase treatment and protein precipitation procedures, about 1 μg DNA was obtained and rehydrated in 50 μl double-distilled water (ddH_2O). The extracted genomic DNA was checked by electrophoresis on a 1.5% agarose gel for quality and quantity.

PCR and restriction. PCR reactions were carried out in 20 μl reaction volumes (1.5 mM $MgCl_2$; 0.2 mM of dATP, dGTP, dTTP, and dCTP; 1 μM of each primer; 1 U *Taq* polymerase (Promega, Madison, WI, USA); and 50 ng of template DNA). The cycling conditions were as follows: 95°C for 5 min for denaturation, 30 cycles for amplification (95°C for 30 s, a sequence specific temperature for 30 s, and 72°C for at most 90 s), and a final extension at 72°C for 10 min. For flies in the crossing experiments, PCR

products were digested with specific enzymes. For the restriction survey, successful PCR products (i.e., showing a sharp single band on a gel) were subjected to restriction enzyme digestions to detect if a sex difference exists in *D. albomicans*. The RFLP marker was named after the PCR locus plus the restriction enzyme, e.g., a10+*Rsa*I. Different restriction fragments were separated on a 2% agarose gel, and the sizes of the DNA fragments were estimated by comparing them to a 100 bp ladder (Bioman, Taipei, Taiwan). Intron size was estimated by comparing the genomic product and the expected size from the cDNA sequence. Sizes of *Amyrel*+*Bgl*II restriction fragments and the *Amyrel* intron were calculated from the published sequence and confirmed on the gel. The sizes of the others were estimated from the electrophoresis gel.

In situ hybridization of polytene chromosomes

Third instar larvae grown at a low density in standard cornmeal medium at 18°C were dissected, and salivary gland chromosomes were prepared for *in situ* hybridization following procedures modified from Montgomery et al. (1987). Larvae were dissected in 45% acetic acid, and the salivary glands were transferred into a mixture of lactic acid, acetic acid, and ddH_2O (1: 3: 2), squashed under a coverslip, and kept overnight at 4°C. The coverslip was then removed after freezing the slide in liquid nitrogen. The probes were synthesized using a PCR DIG Probe Synthesis Kit (Roche, Basel, Switzerland) and checked on an agarose gel. The PCR-synthesized probes were denatured by heating to 90°C for 7 min; polytene chromosomes were denatured by 0.07 N NaOH for 3 min; and a drop of the denatured probe was then added to the denatured chromosome slide and covered with a coverslip. Hybridization procedures followed the protocol of Wang et al. (2004) but used a DIG DNA Labeling and Detection Kit (Roche). Slides were with a microscope at 400x magnification under bright-field for the signal which appeared as a clear dark band on the chromosome; afterwards its location was determined under phase contrast where it appeared as an inconspicuous hollow band. Photomicrographs were taken using a binocular phase-contrast microscope (IX71, Olympus, Tokyo, Japan) with a CCD (charge-coupled device) (DP70, Olympus).

RESULTS

From the crossing experiment, only 3 sex-linked RFLP markers were obtained; 2 *D. albomicans* isofemale strains showed a sexual difference (i.e., homozygous females and heterozygous males). We therefore performed the PCR restriction survey using one of these 2 isofemale strains. Finally, *in situ* hybridization confirmed the hypothetical chromosome arm homologies extrapolated from the previous 2 experiments.

Sex linkage in hybrids

From the *D. albomicans* cDNA library, 1,490

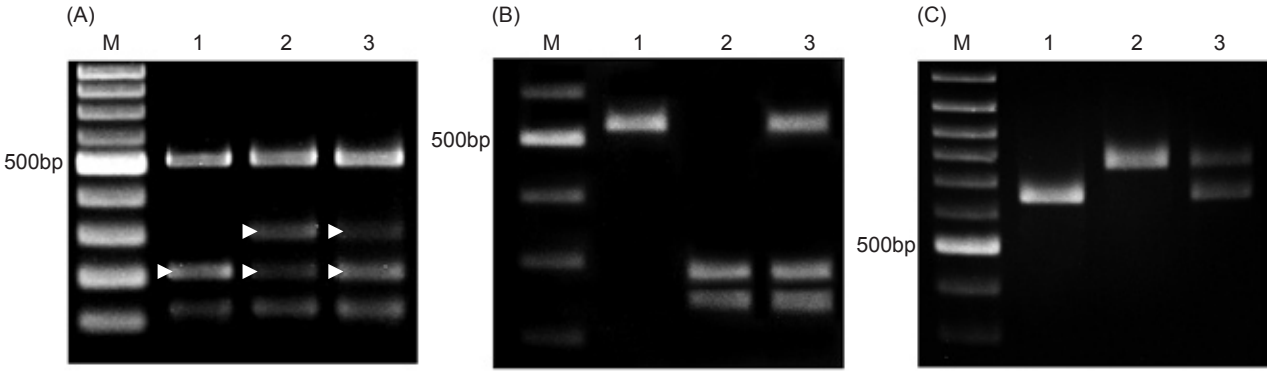

Fig. 2. PCR-RFLP patterns of 3 markers: (A) a10+*Rsa*I, (B) c29+*Rsa*I, and (C) a52+*Hae*III. Lanes 1 and 2 are the homozygotes for 2 different alleles, lane 3 is the heterozygous form, and M is the molecular marker (100 bp ladder). The pattern of a10+*Rsa*I was more complicated than the other 2. The uppermost band was common to all 3 genotypes. The arrowhead indicates the difference in the patterns. The heterozygous pattern can be discriminated from the homozygous pattern in lane 2 by contrasting the intensity of the 2 intermediate bands (arrowheads).

clones were sequenced, and after excluding 26 mitochondrial sequences, 830 nuclear loci were obtained by assembly with the subroutine SeqMan of the program DNA*. From the *D. nasuta* cDNA library, 134 clones were sequenced, and after excluding 3 mitochondrial sequences, 112 nuclear loci were obtained. Thirty-six loci were found to be homologous in both libraries (unpubl. data). Five of the shared loci with a proper interspecific restriction difference were chosen for primer design and used as RFLP markers for the crossing experiment.

From the 2 sets of crosses (#296.6 ♀ × #252.11 ♂ and #163.5 ♀ × #193.7 ♂) 3 RFLP markers showed sex linkage in the hybrids. The PCR primers and expected product sizes according to the cDNA sequences are listed in table 1, and restriction patterns for each of the 3 genetic markers, a10+*Rsa*I, c29+*Rsa*I, and a52+*Hae*III, are shown in figure 2. Due to strain variations, not all 3 markers were adequate for both crosses. Two markers (c29+*Rsa*I and a10+*Rsa*I) were used

Table 1. Primers and expected cDNA sizes of chosen loci for all 3 experiments

Locus	Primer pairs	cDNA length (bp)	Experiments[a]
a10	F: GTACGATGGCAACCTCTTCGCACC R: CCCATCTTATTGAGAGCGCAGCAC	389	1
a28	F: GGGGCACACTGATTTATTAAACAAGC R: TATTTTACGCCACAACTTGCAGCAC	515	3
a52	F: TATTCATCGCATTCCACAT R: GGCTTCCTCAATCAACTG	501	1, 3
a70	F: GGTATCGGATCAGTATTCGGCTCC R: GTTATCAGTACCCGCCACAATAAATTC	973	3
a78	F: GGGATATTGTAGAAAAGCTTGAAGCTG R: GTATGTTTGTCCCTACAAGGGTTGC	390	3
a286	F: CTGCCACCACCATCGGAAG R: CAGCCAATACCCCCAGGAA	587	3
a386	F: GTTACGATTACGAAGAGTGC R: CTGCCGTGCTTATGTGAT	413	2
a427	F: ATTGTTGTGGGTGCCGTGT R: GAGCAATACTACAAACGCATCG	268	2
a1185	F: ATTCTGTCGTTCGTTTTGA R: GATTTCGGCTTACATTATTG	422	2
a1953	F: GCCAACAGCGAGCCTTCT R: GCGACCCAAGCACGAATC	346	2
a1350	F: GAGGATGACGATGATGTTAG R: CAGAATCGCATACAAGCCCA	422	2
a2160	F: AGCATCAAAGTCGTGTAGA R: AGCAACTCAAACTGGAAC	554	2
a2414	F: GTTTCCCAATGCGTTTAGTA R: ATCCGTCCTATGCTCTATGT	556	2
a2746	F: CGTGGACTTATTTTTCTATG R: AGTGTGTTAGTTACCTGAGC	285	2
Amyrel	F: GGTGGGGAAATCGCAATACG R: GCCATAGCCAAAGTTATCTACGGTC	1343	2, 3
c12	F: CCGAGGGTAATCTGACAT R: GGATGACTGATGCCGTAT	424	3
c29	F: CTGGGCAAAGAGTGTAGG R: CAGAAGGAGGGCGAAAA	530	1
N120H	F: AACAGCCGTCATCTTAGTAG R: AACTTTTCATTGCCCAGACA	558	2, 3
N145H	F: TGCCTGGTGCGGTGGTT R: GCAGACGGTGTTGGTGATGA	391	2

[a]The marker was used in crosses (1), the restriction survey (2), and/or *in situ* hybridization (3).

to check F_2 hybrids from 1 cross, and 2 (c29+*Rsa*I and a52+*Hae*III) were used for the other (Table 2). All F_2 females were homozygous, and males were heterozygous. Sex linkage in the hybrids indicated that these 3 loci were located on the neo-sex chromosomes. The loci homologous to a10 and a52 were located on *D. melanogaster* 3L, and that to c29 on 2R according to the BLAST results (Table 3). Therefore, we hypothesized that the 3rd chromosome arm of the neo-sex chromosome of *D. albomicans* is composed of Muller's elements D and C.

Sexual differences in isofemale strains

We also found that the marker, c29+*Rsa*I, was homozygous in females and heterozygous in males of the #163.5 strain. The other 2 markers, a10+*Rsa*I and a52+*Hae*III, were homozygous in females and heterozygous in males of the #296.6 strain. All 3 genetic markers showed sex linkage in certain *D. albomicans* isofemale strains (Table 4). Sex linkage in *D. albomicans* isofemale strains but not in *D. nasuta* confirmed that these 3 genetic markers are located on the 3rd chromosome arm of the neo-sex chromosome.

Restriction survey of loci with sexual differences in an isofemale strain

Screening based on our arbitrary criteria from the 830 nuclear loci of *D. albomicans* resulted in 136 candidates. These candidate ESTs plus the published *Amyrel* sequence (NCBI) were used for primer design. Only those PCR products with distinctive single bands were digested with restriction enzymes to reveal whether restriction pattern differences existed between males and females of *D. albomicans* strain #296.6. Loci with sexual differences are listed in table 5, and the primers are given in table 1. The *D. melanogaster* homologues of all 11 loci are located on the 2R or 3L chromosome arms (Tables 3 and 5).

Table 2. Single fly PCR restriction patterns of the F_2 flies in 2 crossed sets

Markers	#296.6 ♀ × #252.11 ♂			
	F_2 ♀		F_2 ♂	
	Hetero.	Homo.	Hetero.	Homo.
a10+*Rsa*I	0	11	6	0
c29+*Rsa*I	0	11	6	0
a52+*Hae*III	-	-	-	-

Markers	#163.5 ♀ × #193.7 ♂			
	F_2 ♀		F_2 ♂	
	Hetero.	Homo.	Hetero.	Homo.
a10+*Rsa*I	-	-	-	-
c29+*Rsa*I	0	12	12	0
a52+*Hae*III	0	12	11	0

Hetero., heterozygous; Homo., homozygous.

***In situ* hybridization**

According to the BLAST results, 2 loci on each Muller's element were chosen for *in situ*

Table 3. Homologies suggested by BLAST of *Drosophila albomicans* EST (expressed sequence tags) to *D. melanogaster* loci

Locus	Gene name	Location	*e* value[a]
a10	*RpL28*	3L	3e-64
a28	*Apf*	2L	1e-55
a52	*CG17029*	3L	4e-87
a70	*CG1746*	3R	3e-22
a78	*piwi*	2L	1e-49
a286	*αTry (47F4)*	2R	4e-151
a386	*Pgm*	3L	3e-57
a427	*san*	2R	1e-37
a1185	*CG17280*	2R	5e-61
a1350	*CG18067*	2R	2e-21
a1953	*CG8993*	3L	2e-53
a2160	*NHP2*	3L	3e-68
a2414	*CG5068*	3L	6e-36
a2746	*zetaCOP*	3L	1e-44
Amyrel	*Amyrel*	2R	0
c12	*ninaE*	3R	4e-81
c29	*CG13340*	2R	3e-71
N120H	*Pdh*	3L	1e-76
N145H	*Jon65Aiv*	3L	8e-48

[a]*e* values are from the tblastx method.

Table 4. Single fly PCR restriction patterns in 2 *Drosophila albomicans* isofemale strains

Markers	Strain	♀		♂	
		Homo.	Hetero.	Homo.	Hetero.
a10+*Rsa*I	#296.6	3	0	0	5
c29+*Rsa*I	#163.5	4	0	0	4
a52+*Hae*III	#296.6	8	0	0	4

Hetero., heterozygous; Homo., homozygous

hybridization. The results of *in situ* hybridization (Fig. 3, Table 6) combined with the BLAST results (Table 2) showed that the 2L of *D. melanogaster* is also the 2L of *D. albomicans*, the 3R of *D. melanogaster* is the 2R of *D. albomicans*, and the 2R and 3L of *D. melanogaster* are the proximal 1/2 and distal 1/2, respectively, of the 3rd chromosome arm of the neo-sex chromosome of *D. albomicans*.

DISCUSSION

Drosophila albomicans is unusual among *Drosophila* species in that it has a pair of recently fused neo-sex chromosomes (the 3rd chromosomes fused to the sex chromosomes) (Yu et al. 1999) and therefore only 3 pairs of chromosomes (2m + 1dot), while its sibling species, *D. nasuta*, contains the basic karyotype of the *D. immigrans* group (Wilson et al. 1969). *Drosophila albomicans* is thought to have diverged from *D. nasuta* < 0.5 million yr ago (Chang and Ayala, 1989; Bachtrog, 2006). A gene located on the 3rd chromosome arm is sex-linked in *D. albomicans* but autosomal in *D. nasuta*. Taking advantage of the phenomenon that meiotic recombination does not occur in *Drosophila* males, backcrossing a hybrid F_1 male to the maternal species will indicate

Table 5. Loci showing sexual differences in *Drosophila albomicans* strain #296.6

Locus	RE	Estimated fragment length (bp)	Estimated intron length (bp)
a386	*Acc*I	neo-Y: 480 neo-X: 200, 280	70
a427	*Apo*I	neo-Y: 140, 180 neo-X: 320	50
a1185	*Sty*I	neo-Y: 160, 380 neo-X: 540	120
a1350	*Hpa*II	neo-Y: 170, 250 neo-X: 420	-
a1953	*Dde*I	neo-Y: 410 neo-X: 170, 240	60
a2160	*Ava*I	neo-Y: 130, 490 neo-X: 620	60
a2414	*Dra*I	neo-Y: 610 neo-X: 130, 480	50
a2746	*Bsm*I	neo-Y: 140, 210 neo-X: 350	60
Amyrel[a]	*Bgl*II	neo-Y: 1396 neo-X: 258, 397, 741	53
N120H	*Bsr*I	neo-Y: 90, 580 neo-X: 90, 240, 340	110
N145H	*Hind*III	neo-Y: 470 neo-X: 70, 400	80

RE, restriction enzyme; [a]Sequence data obtained from NCBI.

Table 6. Homologies among chromosome arms of *Drosophila albomicans*, *D. melanogaster*, and Muller's elements

Loci	*D. albomicans* (*in situ*)	*D. melanogaster* (BLAST)	Muller's element
a28 and a78	2L	2L	B
a70 and c12	2R	3R	E
a52 and N120H	distal 1/2 of 3	3L	D
a286 and *Amyrel*	proximal 1/2 of 3	2R	C

whether a gene is located on the 3rd chromosome arm. Laborious studies were performed with 8 morphological markers, and it was determined that one of them is located on the 3rd chromosome (Ashadevi et al. 2005). Codominant molecular markers are much more convenient than ordinary recessive morphological ones to determine sex linkages. We attempted to find some loci with sexual differences as revealed by PCR-RFLP. Although genomic libraries may provide more-informative DNA comparisons of these 2 species for revealing sequence evolution including Y degeneration, we chose a cDNA library approach in order to obtain coding gene sequences for finding homologues in *D. melanogaster*, a distantly related *Drosophila* species. Only 134 clones of a *D. nasuta* cDNA library are not representative of a transcriptome, but were sufficient for us to obtain sequences with interspecific restriction differences. Five RFLP markers were used for genotyping of the traditional crosses, and three of them were confirmed to be located on the 3rd chromosome arm of the neo-sex chromosome of *D. albomicans*. During the crossing experiment, it was found that 3 markers in certain strains of *D. albomicans* were homozygous in all females and heterozygous in all males, but no sexual differences appeared in *D. nasuta*. This is also supporting evidence for these 3 markers being located on the 3rd chromosome arm of the neo-sex chromosome. Through the homologies found by BLAST of these 3 markers of *D. melanogaster*, the 3rd chromosome arm of the neo-sex chromosome of *D. albomicans* was hypothesized to be Muller's elements C and D.

Although an intraspecific difference was not detected in the *D. albomicans* cDNA library, we did observe sexual differences, in certain *D. albomicans* isofemale strains by PCR amplification

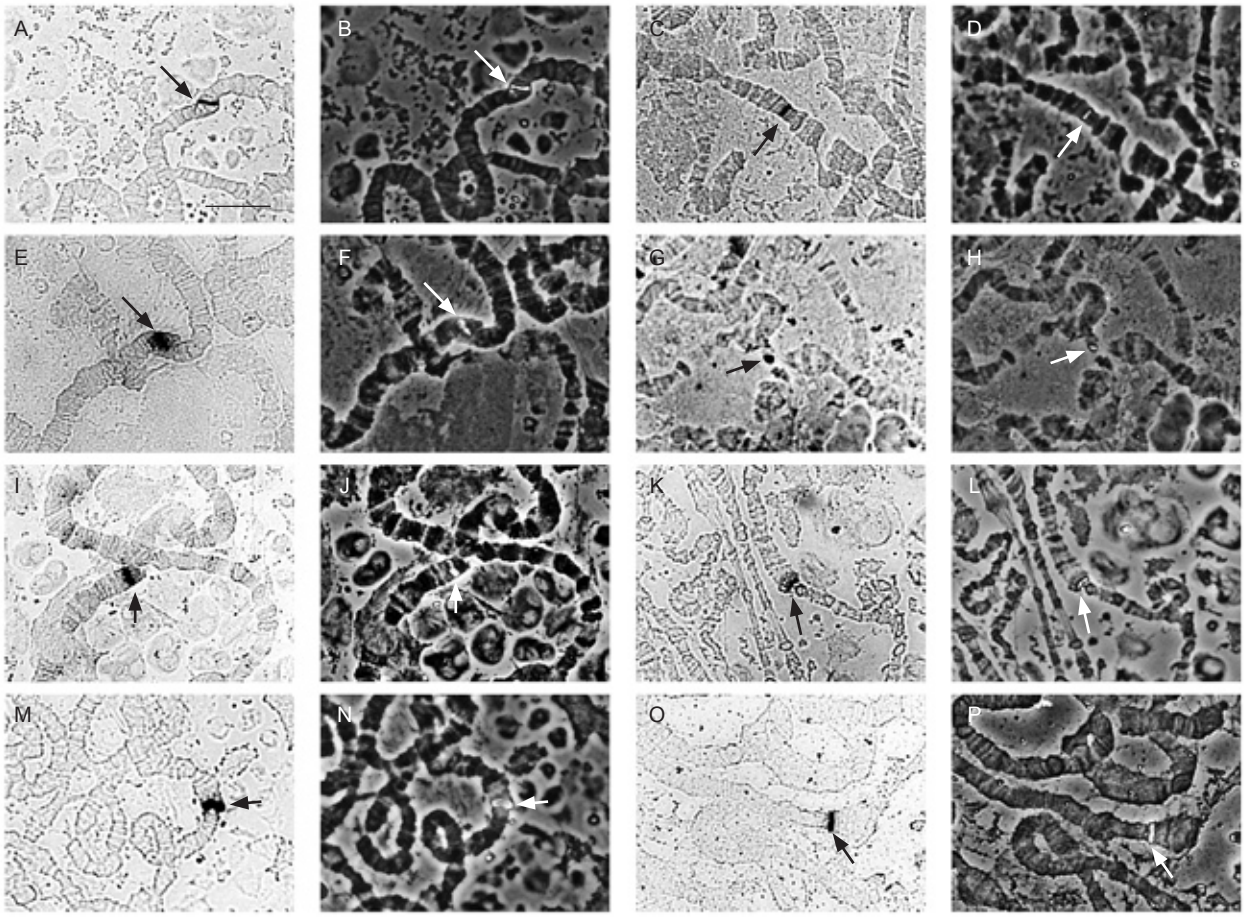

Fig. 3. *In situ* hybridization of salivary gland chromosomes of *Drosophila albomicans*. Loci a28 (A, B) and a78 (C, D) are located on 2L; a70 (E, F) and c12 (G, H) are on 2R. Loci a52 (I, J) and N120H (K, L) are located on the distal 1/2 of the 3rd chromosome arm; a286 (M, N) and *Amyrel* (O, P) are on the proximal 1/2. The arrows indicate the signals on the bright-field photos. Arrowheads indicate the signals on the phase-contrast photos. The scale bar in (A) indicates 20.0 μm, and all pictures are at the same scale.

of sex-linked markers with genomic DNA as the template. This phenomenon showed that it is not necessary to find interspecific sequence differences. It is possible to study sex linkages using sequences from the *D. albomicans* cDNA library alone. More primer sets were designed to survey sexual differences in the #296.6 isofemale strain. Genomic DNAs of a male and a female were both used for the PCR-RFLP survey. Through this approach, another 11 loci, which appeared as homozygous in females and heterozygous in males, were discovered (Table 5). All of these 11 loci have *D. melanogaster* homologues located on Muller's elements C or D. There are other loci also located on Muller's elements C or D but which did not exhibit significant sexual differences. This might have been due to technical limitations of the restriction survey, such as no restriction differences or restriction pattern differences too small to be differentiated on agarose gels. For example, locus a286 with a homologue located on *D. melanogaster* 2R did not show recognizable sexual differences in the restriction survey. However it was revealed to be located on the 3rd chromosome arm of the neo-sex chromosome of *D. albomicans* by *in situ* hybridization (Table 6). No loci with homologues located on *D. melanogaster* 2L or 3R were found to have sexual differences. This provides additional experimental support for the 3rd chromosome of *D. nasuta* consisting of Muller's elements C and D. Fifteen loci (3 from the crosses, 11 from the restriction survey, and 1 from *in situ* hybridization) all matched the expectation with no exceptions, which is strong evidence for the identity of the 3rd chromosome of *D. albomicans* and probably the *D. nasuta* subgroup. Muller's element A is the sex chromosome of both subgenera, *Sophophor*a and *Drosophila*. The assumption that the 3rd chromosome equals elements C and D also implies that the 2nd chromosome could be composed of Muller's elements B and E.

In situ hybridization has been used to establish chromosome homologies in species groups of *Drosophila* such as *obscura* (Segarra et al. 1996, Papaceit et al. 2006) and *repleta* (Ranz et al. 1997). By *in situ* hybridization, we not only confirmed the above hypothetical homologies of the arms but also revealed that Muller's element C was located on the proximal (i.e., near the chromocenter) 1/2 of the 3rd chromosome arm of the neo-sex chromosome, element D is on the distal (i.e., away from the chromocenter) 1/2 of the 3rd, B is on the 2L, and E is on the 2R of *D. albomicans*. Ranz et al. (1999) found that chromosome arms in *Drosophila* are quite conservative according to *in situ* hybridization; therefore, we propose chromosome arm homologies between these 2 lineages through a comparison of *D. melanogaster* and *D. albomicans* (Fig. 4). Even though we did not come across any violation of our hypothesis, it is still possible that some loci may change their location to the other arm due to a pericentric inversion (Ranz et al. 2007).

Probably more species in the subgenus *Drosophila* contain the ancestral karyotype (2*n* = 12), while most species in the subgenus *Sophophora* have fewer chromosomes. Karyotype differences in this group are mainly caused by differential fusions of Muller's elements. The basic karyotype of the *D. immigrans* species group has exactly the same chromosome number (2*n* = 8) as *D. melanogaster*; yet the former (1m + 2t + 1dot) differs from the latter (2m + 1t + 1dot). They both have a pair of telocentric X chromosomes and a pair of dot 4th chromosomes which are most probably Muller's elements A and F, respectively. They both have metacentric 2nd chromosomes. The major difference is the shape of their 3rd chromosomes: telocentric in *D. nasuta* but metacentric in *D. melanogaster*. The telocentric 3rd chromosome was hypothesized to have evolved from a fusion between 2 Muller's elements followed by a pericentric inversion (Meera Rao and Ranganath 1991).

A phylogenetic tree was drawn based on the information from FlyBase (http://flybase.bio.indiana.edu) which is consistent with the phylogeny based on the *Adh* and *Gpdh* sequences (Katoh et al. 2007) and fusion data in Ashburner et al. (2005), plus our *D. albomicans* and *D. nasuta* data (Fig. 5). Among the 17 species of the subgenus *Sophophora* and 10 species of the subgenus *Drosophila* listed in the book, fusions of Muller's elements occurred in 7 *Sophophora* and 3 *Drosophila* species. Four fusion events, A+F, B+C, D+E, and, (D+A)&C are specific to *Sophophora*, and 3, C+E, B+E, and (C+D)+A, are specific to *Drosophila*, whereas 2, C+D and D+A, are shared by both subgenera. The B+C fusion occurred in 4 *Sophophora* species, and the D+E fusion also occurred in 3 of these 4 species. The C+E fusion occurred in only 1 *Drosophila* species. The C+D fusion is shared by 3 *Sophophora* and 4 *Drosophila* species. Based on these 29 species, the B+E fusion in the *Drosophila* lineage is unique and is found only in the *D. immigrans* group.

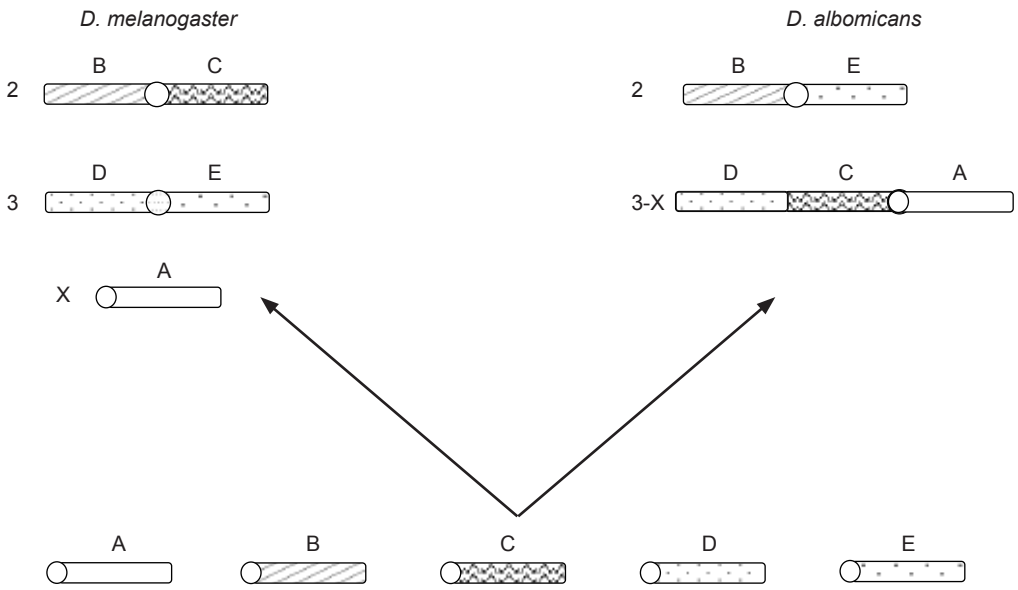

Fig. 4. Four ancestral Muller's elements fused twice to form the metacentric 2nd (B+C) and 3rd (D+E) autosomes in *Drosophila melanogaster*. Muller's element F is not shown. One fusion formed the metacentric 2nd (B+E) autosome, and another fusion between C and D probably followed by a pericentric inversion formed the 3rd (C+D) autosome which later fused with the sex chromosomes and gave rise to the neo-sex chromosomes in *D. albomicans*.

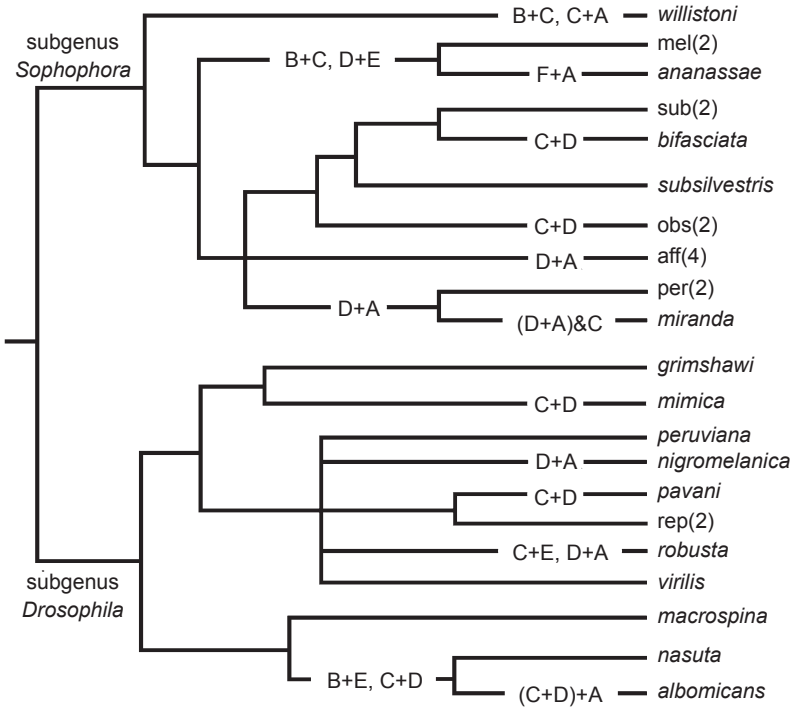

Fig. 5. Phylogenetic tree with fusion events marked on the branches. Fusion data other than from *Drosophila albomicans* and *D. nasuta* were obtained from Ashburner et al. (2005), and the phylogenetic relationship was derived from FlyBase. The fusion of Muller's elements is indicated on the branch. An ampersand (&) was used to designate the special case of only the Y chromosome of the homologous (D+A) pair being involved in the (D+A)&C fusion of *D. miranda*. The branch length is not proportional to the divergence time, and species with the same karyotype are grouped together for simplicity not monophyly. aff (4), *affinis*, *algonquin*, *athabasca*, and *azteca*; mel (2), *melanogaster* and *simulans*; obs (2), *obscura* and *ambigua*; per (2), *persimilis* and *pseudoobscura*; rep (2), *repleta* and *hydei*; sub (2), *subobscura* and *madeirensis*

Although the 2 subgenera share fusions such as C+D, it is clear from the phylogenetic tree that these fusions represent a homoplasy.

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