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Early-Stage Evolution of the Neo-Y Chromosome in *Drosophila albomicans*

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Chia-Hao Cheng, Ching-Ho Chang, and Hwei-yu Chang (2011) Early-stage evolution of the neo-Y chromosome in *Drosophila albomicans*. *Zoological Studies* **50**(3): 338-349. Numerous theories have specified that an originally autosomal neo-Y chromosome arm is expected to undergo degenerative evolution. Neo-sex chromosomes of *Drosophila albomicans* originated from 2 Robertsonian translocation events, one for X and the other for Y, between ancestral *Drosophila* sex chromosomes and a pair of autosomes homologous to the 3rd chromosomes of its sibling species *D. nasuta*. Since the neo-sex chromosome in *D. albomicans* is still evolutionarily young, we used genetic approaches to reveal changes in the entire neo-Y chromosome. Non-disjunction is an indicator used to investigate differences between homologous chromosomes. In this study, we first confirmed that no male recombination had occurred in hybrid males of these 2 sibling species. With the aid of molecular marker genotyping and direct karyotyping of aneuploid offspring produced through specially designed crosses and backcrosses of fertile hybrids, we found that the non-disjunction rate was significantly higher in hybrid males with the neo-Y chromosome than in hybrids without it. The high non-disjunction rate made it possible to generate 3,X,X/neo-Y F_2 females and X,neo-Y/neo-Y F_3 male offspring which can reveal recessive effects of the homozygous 3rd chromosome arm. Results of this aneuploid study revealed severe recessive inviability of the neo-Y chromosome. Our results further suggested that increased non-disjunction in hybrid males with the neo-Y chromosome is likely due to changes that occurred on the Y arm, whereas recessive deleterious alleles might be located on the 3rd arm of the neo-Y chromosome. Taken together, the elevated non-disjunction rate and severe recessive inviability revealed significant changes in the neo-Y chromosome at this early stage of chromosome evolution in *D. albomicans*. http://zoolstud.sinica.edu.tw/Journals/50.3/338.pdf

Key words: Meiosis, Neo-sex chromosome, Non-disjunction, Recessive deleterious allele.

Neo-sex chromosomes which formed by fusion events between sex chromosomes and autosomes have independently evolved in many *Drosophila* lineages. Due to achiasmatic meiosis found in male *Drosophila* (Morgan 1912), the neo-Y chromosome which only exists in males might lack recombination after fused chromosomes being fixed in the population. A non-recombining neo-Y chromosome was predicted to degenerate due to Muller's ratchet, background selection, the Hill-Robertson effect, and selective sweep (Charlesworth and Charlesworth 2000). For example, the neo-Y chromosome of *D. miranda*, the neo-sex chromosomes of which diverged more than 10^6 yr ago (Ma), has accumulated many deleterious mutations and transposable elements (Bachtrog and Charlesworth 2002, Bachtrog 2005). In another case, the neo-Y chromosome of *D. psudoobscura* has lost most of its genes and heterochromatinized since it diverged from the neo-X about 18 Ma (Carvalho and Clark 2005). Although those studies showed obvious

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degeneration due to the lack of recombination in neo-Y chromosomes, early changes after the fixation of neo-sex chromosomes are still unclear. Examining a *Drosophila* species with a younger neo-sex chromosome system would be helpful in answering this question.

Drosophila albomicans has a pair of neo-sex chromosomes and can be crossed with its allopatrically distributed sibling species, *D. nasuta* (Kitagawa et al. 1982). The neo-sex system appears in every *D. albomicans* lineage but is totally absent from other species of the *D. nasuta* subgroup (Meera Rao and Ranganath 1991, Chang et al. 2008). This fact implies that the age of the neo-sex system of *D. albomicans* is even younger than species divergence, which is estimated to have occurred < 0.5 Ma (Chang et al. 1989, Bachtrog 2006). According to homology, we call these 2 fused parts the Y (or X) arm and the 3rd arm of the neo-sex chromosome in this study. Since the divergence period is not sufficiently long, neither the differential substitution rate between the neo-X and neo-Y nor obvious degeneration of the neo-Y chromosome was observed on the 3rd chromosome arms by a sequence analysis (Chang 2008). However, those results could not rule out other changes such as meiotic behavior and an accumulation of deleterious alleles on the entire neo-sex chromosome. In our previous study, a free 3rd autosome (i.e., not fused to a sex chromosome) acquired from *D. nasuta* was fixed in certain hybrid strains and behaved like a Y chromosome without recombination (Chang and Kung 2008). Homozygotes of this chromosome, which was designated a Y-like chromosome, can be obtained by backcrossing males from this strain to *D. nasuta*. Our study revealed that the fitness of individuals carrying homozygous Y-like chromosomes began to decline within a few hundred generations (Chang and Kung 2008). Therefore, the 3rd chromosome arm of the neo-Y in *D. albomicans* is expected to degenerate due to the accumulation of recessive deleterious alleles after millions of generations without recombination.

Accordingly, we suspect that examining changes at the chromosome level may be more accessible than averaging substitution rates or finding non-functional evidence from randomly chosen genes on the chromosome at this early stage of evolution of the neo-Y chromosome. In principle, the accumulation of recessive deleterious alleles on the neo-Y chromosome could be examined by the survival rate of individuals homozygous for the neo-Y. However, a YY individual cannot survive in an XY sex-determination system. Instead, non-disjunction, an abnormal whole-chromosome behavior, may serve as an indicator to reveal the divergence among sex-related chromosomes. Aneuploids with an abnormal number of ancestral-type sex chromosomes (i.e., X or Y chromosome of *D. nasuta*) caused by non-disjunction are frequently found in hybrid populations derived from these 2 sibling species (Yu et al. 1999). This non-disjunction may indicate mispairing between these inter-specific chromosomes. Although the pairing of homologous chromosomes, which is essential for segregating them into 2 separate gametes, is a nearly universal feature of sexual reproduction, non-disjunction was discovered in *D. melanogaster* nearly 100 yr ago (Bridge 1913 1916). This non-disjunction can be used as a special tool for different genetic analyses; for instance, it may generate X,neo-Y/neo-Y individuals which could provide a new chance to investigate recessive inferiority of the neo-Y of *D. albomicans*.

Genetic markers can serve as fundamental tools to reveal the non-disjunction rate of hybrids and the accumulation of recessive deleterious alleles. We previously established several molecular markers (Chang et al. 2008) which can be used for easy and rapid detection of hybrid non-disjunction under the prerequisite of "no male recombination". No crossing-over during meiosis in male *D. melanogaster* of the subgenus *Sophophora* was first reported by Morgan (1912), and achiasmatic meiosis is characteristic of nearly all higher dipteran males, like *Drosophila* (Gethmann 1988). However, even in the *Sophophora* subgenus, an appreciable level of spontaneous male recombination was discovered in *D. ananassae* (Kikkawa 1938). Since *D. albomicans* is classified into the subgenus *Drosophila* which diverged from *Sophophora* more than 60 Ma (Beverley and Wilson 1984, Tamura et al. 2004), whether it also lacks male recombination requires experimental support.

In this study, genetic experiments were conducted to understand this early stage of evolution of the neo-sex chromosome by investigating hybrid offspring of these 2 sibling species. Reciprocal crosses between *D. albomicans* and *D. nasuta* can produce 3 kinds of (2 male and 1 female) hybrids with different sex-related chromosome configurations (3,X/neo-Y; 3,Y/neo-X; and 3,X/neo-X in Fig. 1). The non-disjunction rate of these hybrids can be estimated by aneuploid offspring produced through specially

designed crossing schemes with proper genotyping or karyotyping. In addition, the backcross of a 3,X/neo-Y male to a *D. nasuta* female can produce 3,X/X,neo-Y female offspring due to non-disjunction. Afterwards, backcrossing these aneuploid females to *D. albomicans* males may produce X,neo-Y/neo-Y males homozygous for the 3rd chromosome arm. Through these genetic approaches, we were able to reveal the existence of recessive deleterious alleles on the neo-Y chromosome if those XYY males were absent or rare.

MATERIALS AND METHODS

Drosophila strains and crossing schemes

Inbred strains *D. albomicans* #163.5-IA and *D. nasuta* #304.141-IA were derived from #163.5 (Okinawa, Japan) and #304.141 (Mauritius) respectively. These 2 highly inbred strains established by 10 generations of single-pair sib-matings from isofemale strains were a gift from Prof. Chau-Ti Ting of the Department of Life Science, National Taiwan Univ. (Taipei, Taiwan). The #163.5-IA strain has a marker to distinguish the neo-sex chromosomes from the 3rd chromosome of *D. nasuta*. In addition, 2 isofemale strains, *D. nasuta* strain #252.11 from India and *D. albomicans* strain #254.29 from Thailand, were also used in this study. The #254.29 strain was chosen for its special neo-Y marker. Flies were maintained as previously described (Yu et al. 1999, Chang and Tai 2007). All crosses were made using 4-d-old virgin flies, and the flies were sexed within 8 h after emergence.

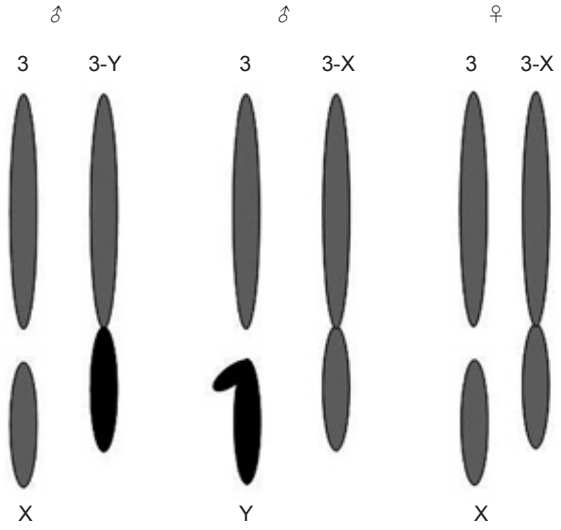

Fig. 1. Diagram of sex-related chromosome configurations in 3 kinds of (2 male and 1 female) hybrids produced by reciprocal crosses between *Drosophila albomicans* and *D. nasuta*.

In this study, crossing schemes 1 to 6 were designed to determine the recombination rates of sex chromosomes in *D. albomicans* (1 and 2) and non-disjunction rates in hybrid offspring (3, 4, and 5), and to detect recessive deleterious alleles on the neo-Y chromosome (6). Two separate pairs of each crossing scheme were established in the beginning as 2 repeats instead of generating repeats in subsequent generations. In general, 20 offspring were genotyped as 1 sample.

Molecular markers

Four loci with inter-specific differences between *D. albomicans* strain #163.5-IA and *D. nasuta* strain #304.141-IA were chosen to analyze the recombination rate. Locus a1350 is located at the proximal end and N120H at the distal end of the 3rd chromosome arm, while 2 autosomal markers, a28 and a70, are respectively located on the 2L and 2R chromosome arms (Chang et al. 2008). Locus information and primer sequences designed for the polymerase chain reaction (PCR) are listed in Chang et al. (2008).

Marker a1350 is able to distinguish the 3rd chromosome of *D. nasuta* from the neo-sex chromosomes of the *D. albomicans* #163.5-IA strain, but cannot discriminate the neo-X from the neo-Y chromosome. Another marker, *Amyrel*, was used to distinguish the neo-Y chromosome of *D. albomicans* strain #254.29 from both the neo-X of the same strain and the 3rd chromosome of *D. nasuta*, but it was unable to distinguish the latter 2. Therefore, marker a1350 was suitable for estimating non-disjunction of F_1 hybrid males but not hybrid females from crosses using strain #163.5-IA males. On the other hand, marker *Amyrel* was able to discriminate the neo-Y allele of *D. albomicans* strain #254.29 which has a 206-base pair (bp) deletion, so we used this marker to detect non-disjunction of both F_1 hybrid males and females through a crossing scheme using males of this strain.

Genotyping

Single-fly genomic DNA extraction. Genomic DNA of a single fly was extracted using the Puregene Cell and Tissue DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA). Each fly was homogenized in 100 μl of a cell lysis solution.

After RNase and protease treatments, alcohol-precipitated DNA was rehydrated in 20 μl of a hydration buffer solution (Gentra Systems). The quality of the isolated genomic DNA was checked by electrophoresis on a 1.5% agarose gel.

PCR and restriction. PCRs were carried out in 20 μl reaction volumes (1.5 mM $MgCl_2$, equal parts of 1 mM dATP, dGTP, dCTP, and dTTP, 0.1 mM of each primer, 2 μl 10x buffer (Invitrogen, Carlsbad, CA, USA), 0.2 μl *Taq* polymerase (Invitrogen), and 1 μl of genomic DNA solution). The cycling standard program was as follows: 95°C for 5 min for denaturation, 35 cycles for amplification (95°C for 30 s, a sequence-specific temperature for 30 s, and 72°C for 50 s), and a final extension at 72°C for 10 min. The annealing temperature was 51°C for marker a1350, 52°C for N120H, and 62°C for *Amyrel*. The PCR product of *Amyrel* did not need further restriction because different alleles had a distinctive size difference. For those without a size difference, such as a1350 and N120H, the PCR products were digested by the respective restriction enzymes, *Hpa*II and *Bgl*II, prior to agarose gel electrophoresis.

PCR sequencing. For markers without restriction differences, such as a70 and a28, genotyping was carried out through the direct sequencing of the PCR products. PCR annealing temperatures were 54°C for a70 and 57°C for a28. An aliquot of 5.0 μl of the PCR product of a70 or a28 together with 0.05 μl of Exonuclease I (20 U/μl, New England Biolabs, Ipswich, Massachusetts, USA), 0.1 μl shrimp alkaline phosphatase (SAP, 1 U/μl, Roche, Penzberg, Upper Bavaria, Germany), and 0.85 μl double-distilled (dd)H_2O was transferred into a 0.2 ml tube. After incubation at 37°C for 30 min, 80°C for 15 min, and cooling to 4°C, 5.0 μl of ddH_2O and 1 μl of 10 mM primer were added to the reaction mixture to make a total volume of 12 μl. Then, DNA sequencing was performed with an ABI_3730 sequencer (Applied Biosystems, Foster City, California, USA). The forward and reverse strands were assembled by SeqMan® software Version 8.0.2 of DNAstar® (Madison, Wisconsin, USA). Finally, the abl files were double-checked manually.

Karyotyping

Karyotyping was performed as previously described (Yu et al. 1997). F_2 larvae were karyotyped, and the non-disjunction rate of the hybrid F_1 was estimated by the proportion of aneuploids.

RESULTS

Lack of meiotic recombination in *Drosophila albomicans* males

With the aid of a PCR and restriction genotyping analysis, markers a1350 and N120H were used to distinguish the *D. albomicans* type, *D. nasuta* type, and their hybrid type without ambiguity (Fig. 2). For markers which could not be identified by a restriction analysis, such as a70 and a28, a sequencing technique was adopted to show single-nucleotide differences among the *D. albomicans* type, *D. nasuta* type, and their hybrid type (Fig. 3). To check the recombination rates, hybrids from the cross between *D. albomicans* #163.5-IA females and *D. nasuta* #304.141-IA males were backcrossed to *D. albomicans*, and their F_2 offspring were harvested and subjected to a genotype analysis (Scheme 1). Female

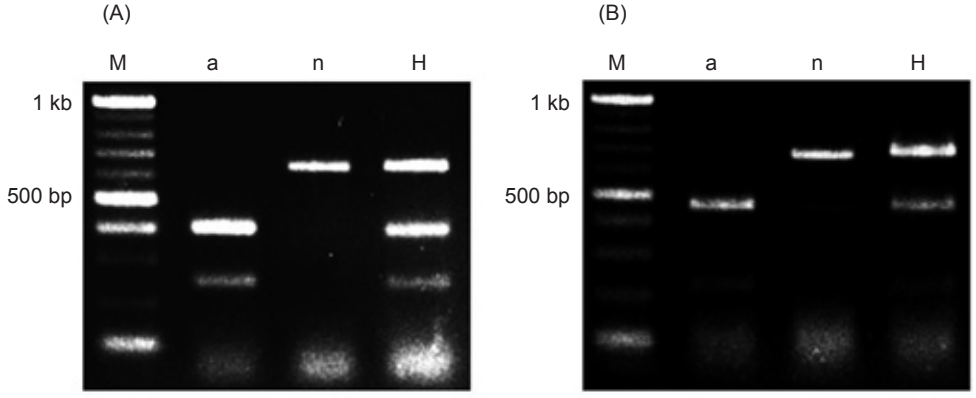

Fig. 2. PCR-RFLP patterns of 2 markers: (A) a1350 + *Hpa*II and (B) N120H + *Bgl*II restriction patterns of *Drosophila albomicans* (a), *D. nasuta* (n), and their hybrids (H) are shown. M is 100-bp DNA ladder; 1 kb and 500 bp are indicated.

recombination rates were 0.13 (n = 40) for the 2 markers (a70 and a28) on the 2nd chromosome (Table 1). A rough estimation of the genetic distance between these 2 markers on the 2nd chromosome is that it is 13 centiMorgans. Since the 3rd chromosome arm consists of 40% of the genome which means that this arm is much longer than 50 centiMorgans, marker a1350 located near the centromere and N120H near the telomere should behave as if they are under independent assortment. The female recombination rate between these 2 markers on the neo-sex chromosomes was 0.48 (n = 40) (Table 1), which statistically fits the expectation. As expected, the male recombination rates of the 2nd autosome and neo-sex chromosomes were both 0 (n = 40) (Table 1).

As illustrated in scheme 2, F_2 males from the

$A_aB_a/A_aB_a \times A_nB_n/A_nB_n$

↓

$A_aB_a/A_aB_a \times A_aB_a/A_nB_n$

↓

Parental: A_aB_a/A_aB_a; A_aB_a/A_nB_n

Recombinant: A_aB_a/A_aB_n; A_aB_a/A_nB_a

Scheme 1. F_1 hybrids from a cross between *Drosophila albomicans* females and *D. nasuta* males were backcrossed to *D. albomicans*. *A* and *B* are 2 loci with inter-specific differences which could be revealed by molecular markers. Subscripts *a* or *n* indicate that the allele is respectively from *D. albomicans* or *D. nasuta*. Parental and recombinant genotypes in F_2 could be revealed by these molecular markers.

Dalb 3^a-X/3^a-X ♀ × Dnas $3^n/3^n$; X/Y ♂

↓

Dalb 3^a-X/3^a-X ♀ × 3^a-X/3^n,Y ♂

↓

♀ : 3^a-X/3^a-X

♂ : 3^a-X/3^n,Y

Scheme 2. F_1 males from a cross between *Drosophila albomicans* (Dalb) females and *D. nasuta* (Dnas) males were backcrossed to *D. albomicans* females. For no male recombination, all F_2 females were homozygous, and all F_2 males were heterozygous for any marker on the 3rd chromosome arm.

Dnas $3^n/3^n$; X/X × Dalb 3^a-X/3^a-Y ♂

↓

Dnas $3^n/3^n$; X/X ♀ × 3^n,X/3^a-Y ♂

↓

Normal F_2 $3^n/3^n$; X/X ♀ 3^n,X/3^a-Y ♂

Non-disjunct F_2 3^n,X/X,3^a-Y ♀ 3^n,X/3^n ♂

Scheme 3. F_1 males from a cross between *Drosophila nasuta* (Dnas) females and *D. albomicans* (Dalb) males were backcrossed to *D. nasuta* females. With a marker with an inter-specific difference or a neo-Y marker, XXY females and XO males produced by non-disjunction could be detected.

Dnas $3^n/3^n$; X/X ♀ × Dalb 3^a-X/3^a-Y ♂

↓

Dalb 3^a-X/3^a-Y ♂ × 3^n,X/3^a-X ♀

↓

Normal F_2 3^a-X/3^a-X ♀ 3^a-Y/3^a-X ♂

3^a-X/3^n,X ♀ 3^a-Y/3^n,X ♂

Non-disjunct F_2 3^a-Y/3^a-X,X ♀ 3^a-X/3^n ♂

Scheme 4. F_1 females from a cross between *Drosophila nasuta* (Dnas) females and *D. albomicans* (Dalb) males were backcrossed to *D. albomicans* males. Non-disjunction may have caused XXY females to have a neo-Y marker and XO males to lack it.

Dalb 3^a-X/3^a-X ♀ × Dnas $3^n/3^n$; X/Y ♂

↓

Dalb 3^a-X/3^a-X ♀ × 3^a-X/3^n,Y ♂

↓

Normal F_2 3^a-X/3^a-X ♀ 3^a-X/3^n,Y ♂

Non-disjunct F_2 3^a-X/3^a-X,Y ♀ 3^a-X/3^n ♂

Scheme 5. F_1 males from a cross between *Drosophila albomicans* (Dalb) females and *D. nasuta* (Dnas) males were backcrossed to *D. albomicans* females. Since no molecular marker could distinguish offspring derived from non-disjunction, XXY females and XO males could only be detected by karyotyping.

Dnas $3^n/3^n$; X/X ♀ × Dalb 3^a-X/3^a-Y ♂

↓

Dnas $3^n/3^n$; X/X ♀ × 3^n,X/3^a-Y ♂

↓

Dnas $3^n/3^n$; X/Y ♂ × 3^n,X/X,3^a-Y × Dalb 3^a-X/3^a-Y ♂

↓ or ↓

3^n,Y/X,3^a-Y ♂ 3^n,X/X,3^a-Y ♀ X,3^a-Y/3^a-Y ♂ X,3^a-Y/3^a-X ♀

$3^n/3^n$; X/Y ♂ $3^n/3^n$; X/X ♀ 3^n,X/3^a-Y ♂ 3^n,X/3^a-X ♀

Scheme 6. F_1 males from a cross between *Drosophila nasuta* (Dnas) females and *D. albomicans* (Dalb) males produced heterozygous XXY F_2 females. One set of F_2 females was further backcrossed to *D. nasuta* males and another set to *D. albomicans* males. After they had produced offspring, those F_2 females were genotyped, and offspring larvae of heterozygous females were karyotyped to check the proportion of XYY males.

backcross of F_1 males should be heterozygous, whereas F_2 females should be homozygous for a sex-linked marker. According to the markers located on the 3rd chromosome arm of the *D. albomicans* neo-sex chromosome, all 20 F_2 males were found to be heterozygous, and all females were homozygous as expected. As a control, the heterozygosity of the autosomal markers of F_2 male offspring was 0.475 (n = 40), and that for F_2 females was 0.50 (n = 40). The autosomal markers as expected were not sex biased whereas the markers on the 3rd chromosome arm showed complete sex-linkage. Our results, which showed an absence of recombinants among F_2 offspring from F_1 males, are consistent with *D. albomicans* having no male recombination, and this holds true for both sex chromosomes and autosomes.

Determination of the non-disjunction rate in F_1 hybrids from different strains of *D. nasuta* females and *D. albomicans* males by genotyping female offspring

To explore the non-disjunction rate in hybrids produced by *D. nasuta* females and *D. albomicans* males, we performed several crosses between different strains of *D. albomicans* and *D. nasuta*, i.e., 2 strains of *D. albomicans* (#163.5-IA and #254.29) and 2 strains of *D. nasuta* (#304.141-IA and #252.11). Four crosses (A, B, C, and D) are described in table 2. The non-disjunction rates of F_1 hybrid males were estimated by the frequency of aneuploids among the F_2 offspring as illustrated in scheme 3. Each experiment began with 2 replicates, and the sample size was 20 F_2 individuals. The aneuploids in F_2 females and F_2 males were revealed using either the a1350

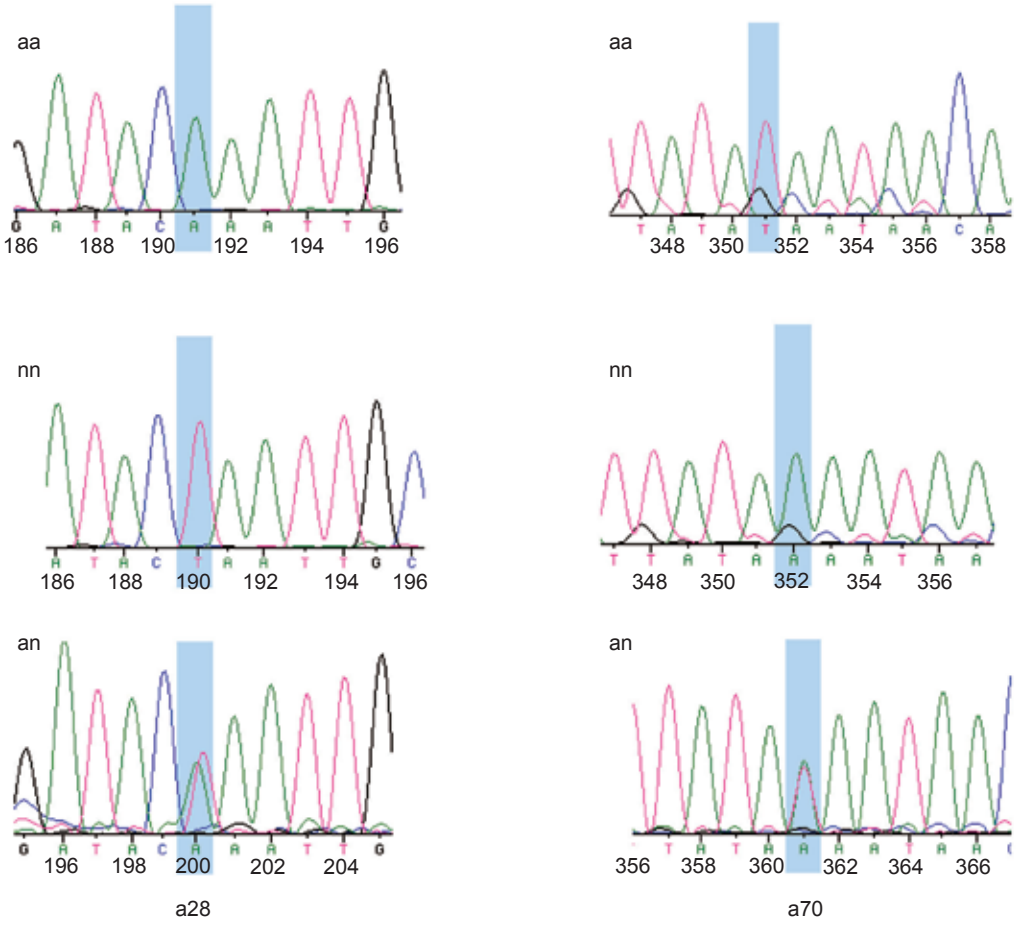

Fig. 3. Inter-specific single nucleotide differences of a28 (left) and a70 (right) among *Drosophila albomicans* (aa), *D. nasuta* (nn), and their hybrids (an) are shaded within a short sequence.

marker for the 3rd chromosome in crosses A and C or the *Amyrel* marker for the neo-Y chromosome in crosses B and D. The *Amyrel* marker on the neo-Y chromosome had a 206-bp deletion which could easily be distinguished from that on the 3rd autosome of *D. nasuta* and that on the neo-X in *D. albomicans* (Fig. 4). Figure 4 summarizes the non-disjunction rates of F_1 hybrid males respectively revealed by F_2 females and F_2 males. The non-disjunction rates revealed by F_2 females showed no statistically significant difference among crosses using different strains. However, non-disjunction rates revealed by F_2 males were lower and more variable than those revealed by F_2 females. Therefore, non-disjunction rates in this study were mainly analyzed using F_2 female data. Furthermore, we also confirmed that XXY females were fertile, whereas XO males were sterile, because no 17 XO males produced offspring when each of them was separately crossed to 3 *D. nasuta* females. As a control, 23 normal males all produced offspring under exactly the same condition.

As illustrated in scheme 4, non-disjunction of hybrid F_1 females with properly designed crosses was detected by the presence of the *Amyrel* neo-Y marker among F_2 females and the absence of this marker among F_2 males in crosses B and D. As shown in table 3, the non-disjunction rate was < 0.05 as revealed by F_2 females. Non-disjunction in hybrid F_1 females (around 0%) was significantly lower than that (around 60%) of F_1 hybrid males.

Table 1. Male and female recombination rates for autosomal and sex-linked markers (sample size = 40)

markers	male	female
autosomal	0	0.13
sex-linked	0	0.48

Table 2. Six combinations of crosses between *Drosophila nasuta* and *D. albomicans* strains

	Male		
Female	*D. albomicans* #163.5-IA	*D. albomicans* #254.29	*D. nasuta* #252.11
D. nasuta #304.141-IA	A	B	-
D. nasuta #252.11	C	D	-
D. albomicans #254.29	-	-	E
D. albomicans #163.5-IA	-	-	F

Increased non-disjunction rates in hybrids with the neo-Y chromosome

No molecular marker was able to reveal the non-disjunction rate of F_1 hybrid males from the other one of reciprocal crosses, i.e., *D. albomicans* females and *D. nasuta* males. Since the 3rd chromosome and neo-X chromosome can only be distinguished cytologically, we analyzed the karyotype of 150 F_2 larvae obtained according to scheme 5. According to our pretest of the karyotypes of these fly stocks, several individuals with an extra Y chromosome were found in *D. nasuta* #304.141-IA; therefore, only *D. nasuta* strain #252.11 was used to conduct this experiment (i.e., crosses E and F in Table 2). The respective frequencies of aneuploids in crosses E and F were 0.09 ± 0.01 (n = 70) and 0.03 ± 0.02 (n = 80). There was no significant difference between crosses E and F, and the average was 0.05. Non-

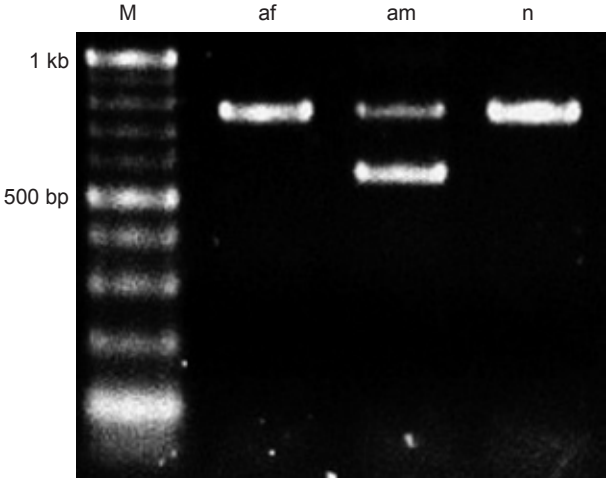

Fig. 4. *Amyrel* PCR patterns of a *Drosophila albomicans* female (af) and male (am), and *D. nasuta* female (n) are shown from left to right. The lower band of the *D. albomicans* male is the neo-Y allele with a 206-bp deletion. M is 100 bp DNA ladder; 1 kb and 500 bp are indicated.

Table 3. Non-disjunction rates of F_1 females revealed by 20 female or 20 male F_2 flies, each consisting of 2 replicates. Crosses B and D are described in table 2

cross	F_2 females	F_2 males
F_1 ♀ of B	0 ± 0	0.05 ± 0.00
F_1 ♀ of D	0 ± 0	0 ± 0

disjunction of these F_1 hybrid males without a neo-Y chromosome was much lower than that of F_1 hybrid males with the neo-Y. These results clearly indicate that it was the neo-Y chromosome which caused the highest non-disjunction rate in hybrids.

Recessive deleterious alleles

The existence of recessive deleterious alleles on the neo-Y chromosome was determined if homozygous neo-Y individuals could not be produced. F_1 males from a cross between *D. nasuta* females and *D. albomicans* males produced aneuploid heterozygous 3,X/X,neo-Y and normal homozygous 3/3;X/X F_2 females (Scheme 3). Homozygous neo-Y offspring were expected from a cross between 3,X/X,neo-Y females and *D. albomicans* males. Two sets of F_2 females were separately crossed to *D. albomicans* and *D. nasuta* males to produce F_3 offspring as illustrated in scheme 6. Crosses to *D. nasuta* males were used as a control. F_2 females were genotyped after they produced F_3 offspring. According to the genotyping results, F_3 offspring larvae from those heterozygous 3,X/X,neo-Y females were karyotyped to determine the proportion of XYY males. Our results showed that not even a single X,neo-Y/neo-Y individual was observed among the 39 larvae produced in the cross of F_2 XXY females with *D. albomicans*, whereas 10 3,X,Y/neo-Y individuals were found among the 47 larvae produced in the cross of F_2 XXY females with *D. nasuta*. The production of XYY F_3 offspring of the 2 crosses with the same type of XXY F_2 females statistically significantly differed ($\chi^2 = 9.26$, $p = 0.002$). Table 4 lists the results of F_3 males only, theoretically an 3,X/X,neo-Y F_2 female could produce 4 kinds of gametes as shown in the left-most column, but the 2 *D. nasuta* X chromosomes were correctly segregated. Therefore, only offspring from the upper 2 kinds of gametes were observed. The absence of X,neo-Y/neo-Y individuals implied that the homozygous neo-Y/neo-Y is probably inviable.

Table 4. Number of hybrids produced by gametes from 3,X/X,neo-Y females and the Y gametes from *Drosophila albomicans* or *D. nasuta* males

	D. albomicans	*D. nasuta*
XXY ♀	neo-Y	3,Y
3,X	9	19
X,neo-Y	0	10
3,X,X	0	0
neo-Y	0	0

DISCUSSION

No male recombination in *Drosophila albomicans* is a prerequisite for an easy and rapid determination of chromosome composition of hybrids and for correct interpretation of neo-Y chromosome evolution. Therefore, the sex-related chromosome configuration in hybrid offspring can be identified by any single marker on it. In addition, predictions of neo-Y evolution will differ if the neo-Y chromosome can recombine with the neo-X chromosome. Due to a lack of recombination by neo-Y chromosomes, the rapid degeneration of the neo-Y chromosomes was observed on non-recombining neo-Y chromosomes in *D. miranda* and *D. pseudoobsura* (Bachtrog and Charlesworth 2002, Bachtrog 2005, Carvalho and Clark 2005, Bachtrog et al. 2008). If recombination occurs on the 3rd chromosome arm of the neo-Y, the genes on this chromosome can be maintained like those on the pseudoautosomal region (PAR) of the human Y chromosome (Simmler et al. 1985). Therefore, we checked before investigating non-disjunction and our data was consistent with no male recombination.

The non-disjunction in hybrid F_1 males from the cross between *D. nasuta* females and *D. albomicans* males produced gametes with 1 more or 1 less X chromosome, and thus generated XXY female or XO male F_2 offspring together with normal XY males and XX females as shown in scheme 3. After normal segregation, the marker on the 3rd chromosome revealed homozygous females and heterozygous males, whereas non-disjunction produced heterozygous (3,X/X,neo-Y) females and homozygous (3/3;X) males. Our strategy of using molecular-marker genotyping to detect non-disjunction is only suitable for the cross mentioned above, and not for reciprocal ones. The reason is that a hybrid offspring with 1 more or 1 less Y chromosome does not change gender, and there are no molecular markers available on the Y chromosome (Scheme 5). However, we adopted karyotyping to detect non-disjunction in crosses E and F (Table 2). Since meiotic recombination does occur in females, we could only cross them to males from the strain with the *Amyrel* marker located on the 3rd chromosome arm of the neo-Y to determine the F_1 female non-disjunction rate.

The average non-disjunction rate of 3,X/neo-Y

hybrid males was estimated to be 0.6, which is higher than the maximum value (< 0.5) suggested by Bridge's model (Bridge 1916). Although Bridges (1916) did observe a higher non-disjunction rate in XXY females compared to normal XX females, this XXY female case differs from the 3,X/neo-Y male case. The 2 X chromosomes failed to undergo crossing over, and the "secondary non-disjunction" proposed by Cooper (1948) directed the segregation of 2 achiasmatic X chromosomes to opposite poles by the Y chromosome in an X-Y-X trivalent (Cooper 1948, Xiang and Hawley 2006). However, the 3/X/neo-Y trivalent cannot be the reason for the high non-disjunction rate in this case as the trivalent in Cooper's case, because the other trivalent 3/Y/neo-X male and trivalent 3/X/neo-X female (Fig. 1) did not have such a high non-disjunction rate. Besides, pairing and recombination of all of these inter-specific 3rd chromosome arms existed, and the evidence will be extrapolated later. The most likely scenario is that the bivalent plus univalent pattern which may have caused 50% non-disjunction is the major pattern in 3,X/neo-Y meiosis pairing, because the X chromosome has difficulty pairing with the Y arm of the neo-Y chromosome. Since we were unable to directly observe non-disjunction, the value 0.60 is just an estimate and could be biased for 2 reasons. The small sample size is a possible cause for a deviation from the expected 0.5, and heterosis is another possibility. If heterozygous 3,X/X,neo-Y individuals grow better than homozygous 3/3;X/X individuals, the observed aneuploid frequency, an indicator of non-disjunction, could increase. Despite the fact that we do not know the exact non-disjunction rate, it is obvious that the rate is high.

Another interesting result is that the non-disjunction rate revealed by male offspring was significantly lower than that revealed by female offspring. This is probably also due to a biased estimate if the survival rate of XO males is lower than that of XY males. Moreover, the sizes of the Y arms of the neo-Y chromosomes differed (Lin et al. 1974) between the 2 strains of *D. albomicans* used in this study, so epistatic defects caused by a lack of the Y chromosome might vary among different genetic backgrounds, which could lead to a high variation in aneuploid males' viability. Since no incompatibility of the autosome was found in this and previous studies (Chang and Kung 2008, Lin et al. 2008), we can infer that the lack of a Y chromosome may display epistatic defects or mis-cooperation of sex chromosomes. Again, estimates of the non-disjunction rate using adult offspring could be biased because of the influence of survival rates. The proportions of aneuploids between replicates were consistent in F_2 females but variable in F_2 males. There is a possibility that the viability of males is more prone to be influenced. The non-disjunction rates of hybrids from different strains showed no discrepancy among the 4 crosses, although we used different markers (Fig. 5). This is important because we have 2 *D. albomicans* strains with different markers, and only strain #254.29 could be used to detect non-disjunction in F_1 females. Since the strain effect was insignificant, data from different crosses can be compared.

Unlike 3,X/neo-Y hybrid males which had a high non-disjunction rate, rates of 3,Y/neo-X hybrid males and of 3,X/neo-X hybrid females were 0.05 (i.e., 7 of 150) and < 0.05 (Table 3), respectively. Both of them were significantly lower than that of 3,X/neo-Y males. Among the 3 kinds of hybrids, only that with a neo-Y chromosome showed an extraordinarily high non-disjunction rate. Obviously, this is not a problem of hybrid males, because hybrid males from the reciprocal cross did not have such a high rate. The high non-disjunction rate implies that the majority of pairing configurations may have been bivalent 3/neo-Y plus univalent X. In other words, the X might not be able to recognize the Y arm of the neo-Y chromosome, and during meiosis, the ancestral X

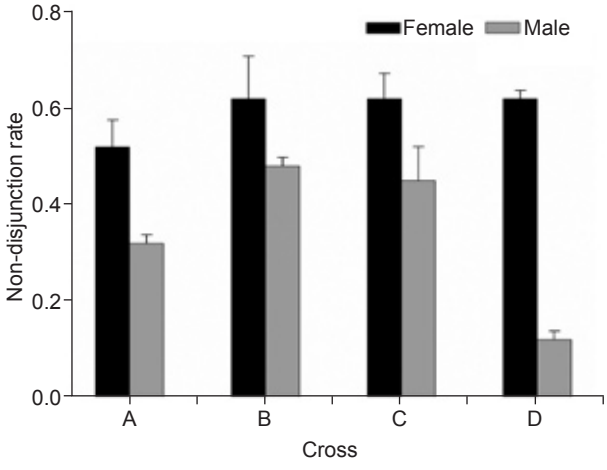

Fig. 5. Non-disjunction rates of young hybrid males from 4 crosses between 2 *Drosophila nasuta* strains and 2 *D. albomicans* strains revealed by F_2 females (black bar) and males (gray bar). Crosses A, B, C, and D are indicated in table 2. No statistically significant differences existed among crosses revealed by F_2 females, but inconsistencies were seen in F_2 male data.

chromosome in the hybrid went to either one of the 2 poles by chance.

In nearly all cases of sexual reproduction, chiasmata during meiosis provide the force to separate homologous chromosomes into 2 separate gametes. While achiasmatic meiosis was observed in *Drosophila* males, chromosome pairing is essential for segregation. In *Drosophila* achiasmatic meiosis, the role of sequence pairing is still unknown, whereas heterochromatic pairing was proven to be associated with homologous segregation (Hughes et al. 2009). One needs to consider the circumstances of both arms. Although pairing of homologous chromosomes is hard to directly detect, crossing over in hybrid 3,X/X,neo-Y females could serve as evidence of it. With the aid of molecular markers, crossing over was observed on both autosomes and the neo-sex chromosomes in XXY females. Intriguingly, we obtained recombinant 3rd chromosomes with homologous exchanged neo-Y termini and neo-Y chromosomes with 3rd chromosome tips in *D. nasuta*. This result revealed that the neo-Y chromosome could regularly segregate with the neo-X chromosome or the 3rd chromosome by pairing with its 3rd chromosome arm. Considering an ordinary *Drosophila* Y chromosome, sequences for recognizing the X chromosome are necessary, and the X chromosomes must have conserved sequences for pairing during meiosis in females (Brianti et al. 2009). In the case of *D. albomicans*, the Y arm of the neo-Y chromosome may be dragged to the correct pole by segregation of the homologous 3rd arm even without paring with the X arm. On the other hand, unlike autosomes, sex chromosomes usually lack large regions of homologous sequences for pairing, so intergenic spacer (IGS) regions of ribosomal (r)DNA and heterochromatic pairing are particularly important in *Drosophila* (McKee 1996). However, in *D. pseudoobscura*, rDNA sequences had disappeared from the Y arm of the neo-Y chromosome (Larracuente et al. 2010). Instead, spreading of IGS sequences in the autosomal arm of its neo-Y was expected to replace the pairing function of the Y arm of the neo-Y chromosome. The condition may be the same in *D. albomicans*, i.e., the pairing region of the Y arm of the neo-Y chromosome was released from functional constraints with the help of the other arm. Interestingly, C banding plus nucleolus organizer region (NOR) patterns, which are associated with the heterochromatic structure and rDNA repeats, showed that the constitution of the Y arm of the neo-Y chromosome extensively differed from that of the Y chromosome of *D. nasuta* (Ranganath and Hägele 1982, Hägele and Ranganath 1983). Comparatively, no significant difference between the 2 species was found in other regions except the dot chromosomes. Moreover, low non-disjunction rates of other hybrids with 3,Y/neo-X illustrate that the Y chromosome of *D. nasuta* could correctly pair with the neo-X chromosome. These phenomena point out the possibility that the Y chromosome of *D. nasuta* may retain ancestral pattern, and the neo-X did not change much either. Therefore, we inferred that the high non-disjunction rate of the hybrid with a neo-Y chromosome might have resulted from changes which occurred on the Y arm of the neo-Y chromosome in *D. albomicans*.

Theoretically X,neo-Y/neo-Y individuals, which were homozygous for the 3rd chromosome arm, can be produced by 3,X/X,neo-Y females. Backcrossing 3,X/X,neo-Y F_2 females to *D. albomicans* males had a theoretical 25% chance of producing X,neo-Y/neo-Y offspring (Scheme 6), because of the production of 3,neo-Y gametes, but none was found among 39 F_3 offspring. Instead of comparing it to the ideal ratio, we compared it with the 21.3% 3,X/Y,neo-Y individuals produced from the same type of females crossed to *D. nasuta* males. The difference between these 2 crosses was significant ($\chi^2 = 9.26$, $p = 0.002$). There are 3 possible explanations for the absence of the expected X,neo-Y/neo-Y individuals (Table 4): (1) chromosome incompatibility; (2) a large X effect; and (3) recessive deleterious alleles. The X-autosome imbalance hypothesis (Muller 1942) and Y chromosome incompatibility (Muller 1942, White 1945) are often used to explain the worse performance of heterogametic F_1 males. Chromosome incompatibility between neo-Y and other chromosomes can be rejected simply by the existence of individuals with the same genetic background except that a 3rd chromosome replaces one of the neo-Y. In addition, F_1 hybrid males with the neo-Y chromosome were viable. As for the 2nd hypothesis, the problem with hybrid males is due to a larger effect of the X chromosome, as the other type of male 3,Y/neo-X containing a larger X chromosome should have more-serious problems but in fact, the contrary was true. Hence, the presence of recessive deleterious alleles on the neo-Y chromosome is the most probable reason. Due to recombination in 3,X/X,neo-Y females, our data can only show the homozygous effect of a limited portion of the neo-Y instead of the entire chromosome, but still

no X,neo-Y/neo-Y offspring were observed. The deleterious allele should be located on the 3rd arm because this arm is truly homozygous, whereas the Y arm is under a situation of XYY, and the hemizygous F_1 was viable.

According to our genetic analysis, no detectable changes were found on the neo-X chromosome, but the high non-disjunction rate and severe inviability suggested that changes had already occurred on the neo-Y chromosome. In *D. albomicans*, the changes might not only have occurred on the 3rd chromosome arm, but also on the ancestral Y chromosome arm. As reported by Larracuente et al. (2010), the entire ancestral Y arm of *D. pseudoobscura* was fused with an autosome, and its ancestral Y-linked locus of rDNA was lost. Large-scale translocations or loss of the rDNA region, which occurred on the ancestral Y arm of the neo-Y, might have been because the pairing regions had escaped from negative selection. The neo-sex systems of these 2 species have the commonality that both ancestral sex chromosomes were fused with a pair of homologous autosomes (Carvalho and Clark 2005, Chang et al. 2008). In addition to the possible changes on the Y arm, evidence of the 3rd arm degeneration in *D. albomicans* was also provided by the absence of X,neo-Y/neoY individuals compared to the existence of 3,X/Y,neo-Y ones (Table 4). Although lacking support from the molecular data, the inferiority of 3,X/neo-Y hybrid males was relaxed in offspring males after being crossed with *D. albomicans*; in other words, defects of the neo-Y chromosome could be covered by the neo-X chromosome (Lin et al. 2008). Numerous recombinant lines obtained from this study will be valuable materials for future studies to map recessive deleterious alleles on the neo-Y chromosome of *D. albomicans*. In conclusion, in the early-stage evolution of the neo-sex chromosomes in *D. albomicans*, we found 2 changes on the neo-Y: (1) the ancestral Y arm may lack the ability for proper meiotic segregation, and (2) there were deleterious alleles on the 3rd chromosome arm.

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