Zoological Studies **47**(3): 293-301 (2008)

Zoological Studies

Cooption of Neo-X and Neo-Y Chromosomes in *Drosophila albomicans*

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(Accepted October 9, 2007)

Sung-Han Lin, Yu-Yang Huang, and Hwei-yu Chang (2008) Cooption of neo-X and neo-Y chromosomes in *Drosophila albomicans*. *Zoological Studies* **47**(3): 293-301. *Drosophila nasuta* has 8 chromosomes, whereas its sibling species *D. albomicans* has 6 because a pair of neo-sex chromosomes has evolved through fusion events in < 0.5 Ma. It remains unclear how the newly joined sex chromosomes differentiated from their homologous 3rd autosome of *D. nasuta*. The body size of F_1 hybrid males from a cross between *D. albomicans* males and *D. nasuta* females was significantly smaller than those of the parental species, but an intermediate size was obtained from the reciprocal cross. There are 2 possible explanations: (1) the ancestral mitochondria of *D. nasuta* are not compatible in the derived *D. albomicans* nuclear environment; and (2) the neo-Y chromosome cannot work well with the homologous ancestral 3rd autosome of *D. nasuta*. In the present study, experiments were conducted to exclude the possible involvement of mitochondrial incompatibility. We established 5 sets of coupled highly inbred *D. albomicans* strains and another 5 sets of *D. nasuta* strains, and subsequently examined their reproductive ability and the body size of their progeny. Each set of coupled strains had nearly the same homogeneous nuclear genome but had different (self vs. non-self) mitochondria. These coupled strains showed indistinguishable reproductive ability and body size between them, indicating that mitochondrial compatibility was not the major cause. Alternatively, our cross experiments demonstrated that the body size of the offspring reverted to normal when the neo-X and neo-Y relationships were restored by backcrossing the small F_1 males to *D. albomicans* females. However, the body size remained small when F_1 males were backcrossed to *D. nasuta*. The backcross results support the 2nd explanation, thus implying that after coevolution of the neo-sex chromosomes, the neo-Y may depend on the presence of the neo-X chromosome in males, but not vice versa. http://zoolstud.sinica.edu.tw/Journals/47.3/293.pdf

Key words: Coevolution, *Drosophila nasuta*, Hybrids, Sex chromosome.

Drosophila albomicans (2*n* = 6) and *D. nasuta* (2*n* = 8) belong to the *D. nasuta* subgroup of the *D. immigrans* species group (Duda 1940, Wilson et al. 1969). According to molecular evidence, this sibling species pair is young and diverged < 0.5 million yrs ago (Ma) (Chang et al. 1989, Bachtrog 2006). During the divergence of these sibling species, hereditary materials in nuclear (n)DNA and mitochondrial (mt)DNA co-evolved for millions of generations within species, as did the X and Y chromosomes. Some aspects of chromosome evolution in this species pair have been studied (Yu et al. 1997 1999, Yang et al. 2004 2008), but many questions remain unanswered.

Except for the larger average body size of adult *D. albomicans* flies comparing to *D. nasuta* under our cultural conditions, these 2 species are morphologically indistinguishable (Chang and Tai 2007). Since body size is a quantitative trait, the hybrid F_1 of reciprocal crosses are supposed to be intermediate. Although female offspring meet this expectation, we noticed that F_1 males from a cross of *D. albomicans* males to *D. nasuta* females were significantly smaller than males of both parental species, whereas the body size of males from the reciprocal cross was intermediate. These observations raised 2 questions: What is the reason for this discrepancy of reciprocal

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crosses and why do only males show this peculiar phenomenon?

Genetic incompatibility is a general phenomenon in hybrids. Our observation regarding the small body size of F_1 males derived from *D. albomicans* males crossed with *D. nasuta* females may be a model for investigating the genetic compatibility between recently divergent species. Considering the difference between males and females, hybrid males and females have nuclei containing genomes derived from both species, while mitochondria are exclusively from the maternal species. Nuclei of hybrid males contain X chromosomes from maternal species and Y chromosomes from paternal species. The body size of males from a specific cross is significantly smaller than even *D. nasuta* whereas the other three of 4 kinds (2 crosses × 2 sexes) of F_1 hybrids showed ordinary quantitative inheritance, and this implies the possibility of abnormal development. Two most probable explanations for this phenomenon are mitochondrial-nuclear relationships and X-Y cooption.

For the 1st explanation, nDNA and mtDNA may individually contribute their influence to the body size of flies, while the interaction between them must also be taken into consideration. Since the effects of mtDNA can be nullified by the complication of the nuclear genetic background, they can only be observed under minimized nuclear variations. We therefore designed a successive backcross scheme between *D. albomicans* and *D. nasuta* to establish coupled strains with the same nDNA but different mtDNA, which would make it possible to demonstrate or exclude the influence of mtDNA without the complication of nDNA. Sex-linked factors are also a possible mechanism causing a reciprocal difference in males. Therefore, the 2nd explanation, X-Y cooption, is even more likely in a species with a pair of large neo-sex chromosomes. By 2 fusion events, the ancestral 3rd chromosomes and sex chromosome pair formed a neo-sex chromosome pair (i.e., a derived character state) in *D. albomicans* (Yu et al. 1999), whereas the 3rd chromosomes remain as autosomes in *D. nasuta*. If the influence of mitochondria can be neglected, the interaction of a neo-Y chromosome with a neo-X chromosome of the same species and that with the 3rd and Y chromosomes of the other species can be compared by backcrossing hybrid F_1 males with both parental species. Backcross experiments would support the neo-Y possibly not functioning well with the 3rd autosome in *D. nasuta* if the body size of the offspring reverted to normal when the neo-X neo-Y relationship was restored.

MATERIALS AND METHODS

All flies were maintained on standard cornmeal medium under a condition of D/L = 12/12 h at 22 ± 1°C and 75% relative humidity.

Drosophila isofemale strains

Two *Drosophila albomicans* strains (#163.5 and #163.18) from Okinawa, Japan and a *D. nasuta* strain (#193.7) from Mysore, India were used in this study. *Drosophila albomicans* strain #163.18 was used to obtain hybrid F_1 and F_2 for measuring body size, while *D. albomicans* strain #163.5 was used to generate coupled strains. *Drosophila nasuta* strain #193.7 was used for both hybrid measurements and generating coupled strains. Prior to the experimental manipulation, the nuclear genomes of all strains were confirmed by their esterase electrophoresis patterns (Yu et al. 1997), and the mtDNA was confirmed by the electrophoresis patterns of the PCR-amplified AT-rich region (unpubl. data).

Crosses and hybrids

Reciprocal crosses between the 2 species, *D. albomicans* and *D. nasuta*, were performed. Hybrids were named Ha_1 and Hn_1 according to the paternal strain. F_1 flies alb_1 and nas_1 were obtained as controls by intraspecific crosses instead of interspecific ones (Fig. 1). F_2 flies produced by backcrossing Ha_1 males to alb_1 and nas_1 females were named AHa_2 and NHa_2, respectively.

Establishment of coupled *D. albomicans* strains with homogeneous nDNA but different mtDNA

Coupled "A" and "a" strains carried nearly the same homogeneous *D. albomicans* nDNA but different mtDNA (i.e., "A" had mtDNA from the same species, but "a" from the other species). Sets of coupled "A" and "a" strains were established by the method illustrated in figure 2. In brief, each set of coupled strains was initiated by 3 flies (i.e., a shared *D. albomicans* male, and a *D. albomicans* and a *D. nasuta* female) only. A *D. albomicans* male separately mated with these 2 females, and only 1 son from the conspecific

D. *albomicans* couple was used to produce 2 lineages of offspring for the next generation (i.e., this male mated with a full sib and a half-sib, respectively). The above procedure was repeated using only 3 flies for each generation until the 10th generation. The resulting 2 closely related and highly inbred strains have been maintained by a non-overlapping generation method since the 11th generation. Five sets of coupled "A,a" strains ("A_1,a_1" to "A_5,a_5") were established. The subscript number indicates different conspecific nuclear genomes for the coupled strains. Coupled strains with the same subscript number contained the same nuclear genome, while "A" indicates the presence of its own mitochondrial genome (i.e., *D*. *albomicans* nDNA with mtDNA), and "a" indicates the presence of the other mitochondrial genome (i.e., *D*. *albomicans* nDNA with *D*. *nasuta* mtDNA).

Establishment of coupled *D. nasuta* strains

The method of establishing coupled "N,n" strains was similar to that of coupled "A,a" strains but using a *D*. *nasuta* male to cross with both a female from the same species and a female from the other species (Fig. 3). The coupled strains had *D*. *nasuta* nDNA but different mtDNA. Five sets of coupled "N,n" strains ("N_1,n_1" to "N_5,n_5") were established. "N" and "n" were designated as described above for "A" and "a" . "N" has *D*. *nasuta* nDNA and mtDNA, while "n" has *D*. *nasuta* nDNA and *D*. *albomicans* mtDNA.

Reproductive ability of the coupled strains

For each set of coupled strains, newly emerged flies were sexed within 8 h and kept in separate vials for 5 d before experiments. Five pairs of 5-d-old flies were put together, and eggs produced from the 24th-96th hour were collected. Numbers of emerging males and females were counted and used as an indicator for the reproductive ability of each strain.

Measurement of wing length

The wing size is positively correlated with the body size of flies (Reeve 1950). Since fly wings are flat and have low water content, after being embedded on slides, wing length was considered to be a proper indicator of body size. The right wing of a fly was dissected, dehydrated with ethanol, and embedded in AQUATEX (Merck, Darmstadt, Germany) as previously described (Chang and Tai 2007). Photos were taken with a digital camera (Coolpix 4500, Nikon, Toyko, Japan) and measured with tpsDig® software (vers. 2.0, F. Rohlf, SUNY, Stony Brook, NY, USA). Wing length was measured following the protocol described in Chang and Tai (2007). In each experiment, the wing length of 100 F_1 flies (i.e., 50 males and 50 females) from Ha_1, Hn_1, alb_1, and nas_1 was measured. In addition to F_1, the wing length of F_2 was also measured in the 2nd experiment with a sample size of 90 males. In coupled inbred strains, the wing length of 20 flies (i.e., 10 males and 10 females) from each strain was measured.

Data and statistical analyses

Either two-way or one-way analysis of variance (ANOVA) was used to analyze wing-length data. Post-hoc Student's *t*-test was used to reveal differences between specific items. In addition, the ordered-heterogeneity test (Rice and Gaines 1994a b) was used to reveal differences in the magnitude of the variance. Data are given as the mean ± the standard error (SE) in the text and tables.

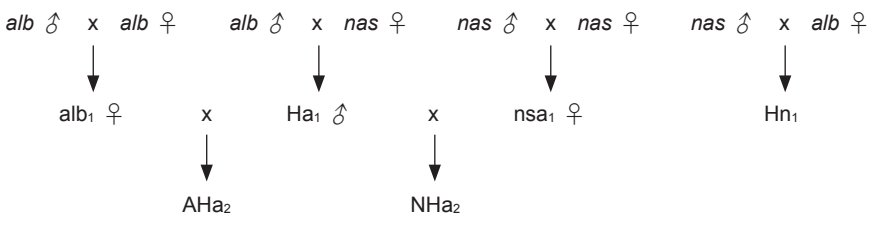

Fig. 1. Hybrid F_1 produced from crossing *Drosophila nasuta* (*nas*) females with *D*. *albomicans* (*alb*) males was named Ha_1, and the reciprocal was named Hn_1. Intraspecific crosses were made by the same procedure as the controls. Ha_1 males were backcrossed to alb_1 and nas_1 females to produce AHa_2 and NHa_2, respectively.

RESULTS

Wing length of hybrids

The results of wing length measurements of *Drosophila albomicans*, *D. nasuta*, and their hybrids are summarized in table 1. By two-way ANOVA, it was determined that body size was significantly influenced by the cross and sex (Table 1). In general, males were significantly smaller than females, and both males and females of *D. albomicans* were larger than those of *D. nasuta*. The size of hybrid F_1 females derived from reciprocal crosses was, as expected, in between those of the 2 species. The size of hybrid Hn_1 males was in between, whereas that of Ha_1 males was significantly smaller than that of *D. nasuta* (nas_1) by post-hoc Student's *t*-test. To supplement the above analysis, we pooled the data of alb_1, Hn_1, and nas_1 males and compared those with Ha_1 males by *t*-test ($p < 0.001$).

Reproductive ability of coupled inbred strains

In order to clarify the interaction between mtDNA and nDNA, we established 5 sets of coupled "A,a" strains and another 5 sets of coupled "N,n" strains. The coupled strains contained the same nDNA but different mtDNA. There was no relationship between the "A,a" and "N,n" strains; for example, both A_1 and N_1 had subscript 1 but had genetic materials from different species. The number of progeny produced by the 5 pairs of flies within 72 h was used to monitor the reproductive ability of each strain, and the average of 5 strains was used to represent each kind of strain (i.e., "A", "a", "N", and "n"). There was no significant difference between strains containing mitochondria of the same and of different species, but a significant difference existed between the "A,a" and the "N,n" strains (Fig. 4). Flies with a nuclear genome from *D. nasuta* produced approximately 2-fold progeny as did those with the *D. albomicans* nuclear genome.

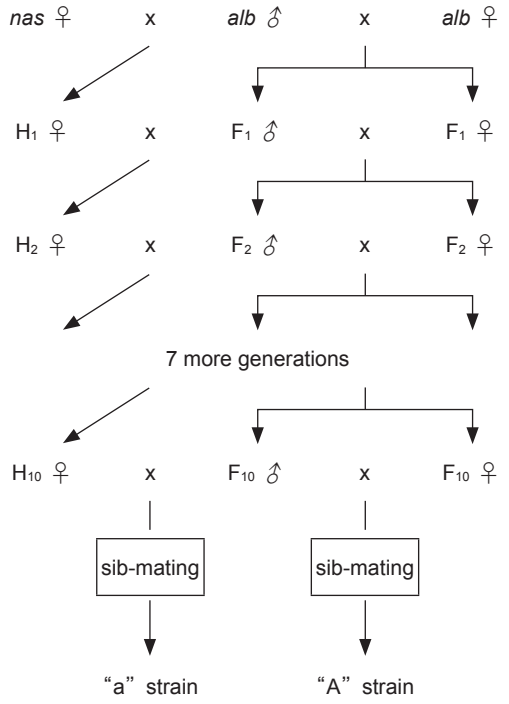

Fig. 2. Coupled strains were initiated with 3 flies (1 male and 2 females). One shared male from the conspecific cross and 1 female from each of the 2 crosses were used to produce the next generation. This 3-fly procedure was repeated until the 10th generation. From the 11th generation, the coupled highly inbred strains were maintained by a non-overlapping generation method as the "A" and "a" strain.

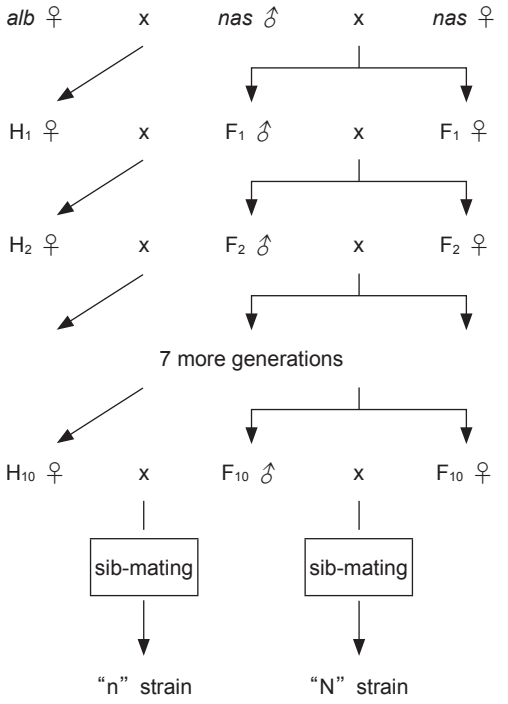

Fig. 3. Coupled strains were initiated by 3 flies (1 male and 2 females). One shared male from the conspecific cross and 1 female from each of the 2 crosses were used to produce the next generation. This 3-fly procedure was repeated until the 10th generation. From the 11th generation, the coupled highly inbred strains were maintained by a non-overlapping generation method as the "N" and "n" strain. The procedure is exactly the same as that in figure 2, except that the 2 species were reversed.

Wing length of coupled inbred strains

Since the body size of males and females significantly differed (Table 1), the wing length of coupled inbred strains of the 2 sexes was analyzed separately (Tables 2, 3). As shown in tables 2 and 3, two-way ANOVA for these 2 factors (i.e., either mtDNA or intraspecific nuclear DNA differences) showed no significant influence of mtDNA either in different genders or under different interspecific nuclear backgrounds ("A,a" And "N,n").

Because no significant influence of mtDNA was detected, the data of coupled strains were combined and designated as "A+a" or "N+n" (Table 4). As with the reproductive ability, a significant difference in body size existed between the "A,a" and "N,n" strains (Table 4).

Backcrosses of Ha_1 males to females of both parental species

To verify whether or not the neo-Y functioned well with the 3rd autosome of *D*. *nasuta*, we backcrossed Ha_1 males to *D*. *nasuta* females. The size of the resulting NHa_2 males (2.35 ± 0.009 mm, n = 90) was similar to that of Ha_1 males (2.35 ± 0.006 mm, n = 90). When Ha_1 males were backcrossed to *D*. *albomicans* females, the resulting AHa_2 males were significantly larger than those of Ha_1 (2.44 ± 0.005 mm, n = 90). One-way ANOVA ($p < 0.001$) and post-hoc Student's t-test showed there to be a significant difference between Ha_1 and AHa_2 but no difference between Ha_1 and NHa_2. Figure 5 shows the wing length distributions of Ha_1, NHa_2, and AHa_2, and the statistical method supported the hypothesis of $\sigma^2_{NHa_2} \geq \sigma^2_{Na_2} \geq \sigma^2_{AHa_2}$ (r_sP_c = 9.998). The increase in variation from Ha_1 to NHa_2 is commonly seen from F_1 to F_2, but the decrease of variation from Ha_1 to AHa_2 indicated that the distribution of Aha_2 was more centralized.

DISCUSSION

Through hybridization of *Drosophila albomicans* and *D*. *nasuta*, we found a significant difference in F_1 male body size between reciprocal crosses. The body size of hybrid females was in between the 2 parental types, as was that of hybrid males from the cross between *D*. *albomicans* females and *D*. *nasuta* males. However, males from the reciprocal cross were significantly smaller than the 2 parental-type males. Under our culture conditions, females of both species were larger than males as usually occurs in insects. *Drosophila albomicans* was larger than *D*. *nasuta*, which is consistent with our previous study (Chang and Tai 2007). Hybrid F_1 males from a *D*. *albomicans* male and a *D*. *nasuta* female were even smaller than *D*. *nasuta* males, whereas F_1 males from the reciprocal cross and F_1 females from both directions were of medium size compared to the parental species. Because the smallest hybrid size was outside of the range of the parental sizes, we considered it to be an abnormal

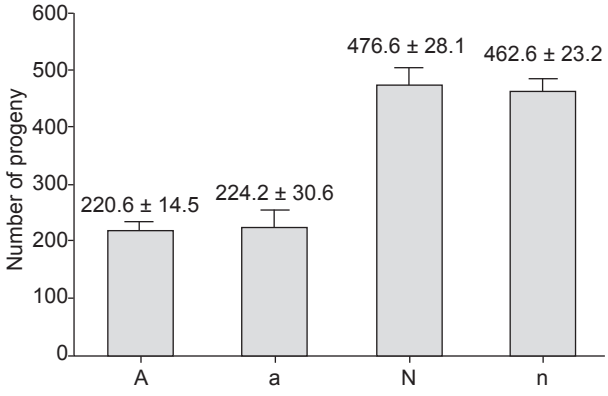

Fig. 4. The number of progeny for each kind of strain is shown by an average (mean ± S.E.) of 5 strains, and the data for each strain are the total offspring produced by 5 pairs of flies in 72 h.

Table 1. Wing length (mean ± S.E. in mm) of *Drosophila albomicans*, *D*. *nasuta*, and their hybrid F_1 with two-way ANOVA results

	alb_1	Ha_1	Hn_1	nas_1
♀	2.75 ± 0.005 (50)[a]	2.72 ± 0.006 (49)	2.71 ± 0.007 (50)	2.68 ± 0.008 (49)
♂	2.40 ± 0.008 (50)	2.34 ± 0.006 (50)	2.38 ± 0.009 (49)	2.36 ± 0.008 (48)

Two-way ANOVA
cross: $F_{d.f.=3}$ = 24.51***; sex: $F_{d.f.=1}$ = 5215.20***; interaction: $F_{d.f.=3}$ = 6.76***

[a]The number in parentheses represents the sample size. *** $p < 0.001$

feature that may have resulted from incompatibility either between mitochondrial and nuclear DNA or between the neo-Y and 3rd autosome.

Several possible mechanisms for asymmetric postmating isolation, such as X-autosome interactions and maternal effects (Turelli and Moyle 2007), can also be applied to explain small hybrid F_1 males from the specific cross between *D*. *albomicans* and *D*. *nasuta*. A maternal effect can be ruled out, because only males showed the smaller body size, while the females were unaffected. Only 2 most likely ones, i.e., the nuclear-cytoplasmic interaction and the neo-Y and 3rd incompatibility, were considered and the reasons are discussed in the following paragraphs.

It has been shown that mitochondrial dysfunction is more serious in hybrid males (Sackton et al. 2003), but this can only explain different results between males and females. *Drosophila albomicans* diverged from *D*. *nasuta* < 0.5 Ma (Chang et al. 1989, Bachtrog 2006), and the karyotype of *D*. *albomicans* is a derived character state while that of *D*. *nasuta* ancestral. In Ha_1 males, the ancestral mitochondria were confronted with a nuclear environment containing a derived genome, while in Hn_1 males from the reciprocal cross, the situation was reversed. The chromosome evolution of *D*. *albomicans* involved 2 fusion events (Yu et al. 1999) and is supposed to change faster. Therefore, it is an interesting assumption that an ancestral mitochondrion cooperates with a derived nucleus with great difficulty, but it is easier for a derived mitochondrion to accommodate an ancestral nucleus.

Interactions between mitochondria and nuclei are inconclusive. *Drosophila simulans* harbors 3 distinct mitochondrial DNA haplotype groups (*si*l, -II, and -III). *si*l haplotype flies of *D*. *simulans* develop the fastest but have the lowest probability of survival despite the different nuclear backgrounds (James and Ballard 2003). In other words, no interaction was evident between mtDNA and nDNA. An opposite conclusion came from *D*. *subobscura*, in which the mtDNA haplotypes of a Finnish strain and a Spanish strain showed advantages over the other under their own respective nuclear backgrounds (Fos et al. 1990). Research evidence has shown asymmetrical interactions between the nuclear background and mitochondria, such as the absolute predominance

Table 2. Wing length (mean ± S.E. in mm) of the "A" and "a" strains for 2 sexes (sample size = 10 flies) and two-way ANOVA results

Coupled strains	Female		Male	
	A	a	A	a
1	2.67 ± 0.05	2.59 ± 0.04	2.39 ± 0.03	2.38 ± 0.02
2	2.55 ± 0.07	2.60 ± 0.05	2.33 ± 0.06	2.38 ± 0.03
3	2.65 ± 0.03	2.66 ± 0.03	2.37 ± 0.06	2.41 ± 0.03
4	2.63 ± 0.04	2.60 ± 0.03	2.41 ± 0.1	2.35 ± 0.03
5	2.61 ± 0.04	2.56 ± 0.03	2.38 ± 0.02	2.34 ± 0.02
Two-way ANOVA				
mtDNA $F_{d.f.=1}$	4.00		0.13	
intra-nDNA $F_{d.f.=4}$	2.93		1.75	
interaction $F_{d.f.=4}$	15.71***		4.94***	

*** $p < 0.001$

Table 3. Wing length (mean ± S.E. in mm) of the "N" and "n" strains for 2 sexes (sample size = 10 flies) and two-way ANOVA results

Coupled strains	Female		Male	
	N	n	N	n
1	2.52 ± 0.03	2.54 ± 0.03	2.25 ± 0.04	2.25 ± 0.03
2	2.54 ± 0.03	2.54 ± 0.03	2.27 ± 0.03	2.25 ± 0.03
3	2.60 ± 0.03	2.56 ± 0.03	2.33 ± 0.09	2.28 ± 0.02
4	2.62 ± 0.02	2.61 ± 0.02	2.33 ± 0.02	2.32 ± 0.01
5	2.54 ± 0.04	2.55 ± 0.03	2.25 ± 0.04	2.28 ± 0.04
Two-way ANOVA				
mtDNA $F_{d.f.=1}$	0.38		1.07	
intra-nDNA $F_{d.f.=4}$	5.54***		7.19***	
Interaction $F_{d.f.=4}$	1.66		2.48	

*** $p < 0.001$

Table 4. Wing length (mean ± S.E. in mm) of 5 "A+a" and 5 "N+n" for 2 sexes (sample size = 20 flies) and two-way ANOVA results

Coupled strains	Female		Male	
	A+a	N+n	A+a	N+n
1	2.63 ± 0.01	2.54 ± 0.00	2.38 ± 0.01	2.25 ± 0.01
2	2.62 ± 0.01	2.60 ± 0.01	2.36 ± 0.01	2.32 ± 0.01
3	2.60 ± 0.02	2.56 ± 0.01	2.39 ± 0.01	2.28 ± 0.01
4	2.61 ± 0.01	2.52 ± 0.02	2.38 ± 0.02	2.25 ± 0.02
5	2.59 ± 0.01	2.56 ± 0.01	2.36 ± 0.01	2.27 ± 0.00
Two-way ANOVA				
inter-nDNA $F_{d.f.=1}$	50.93***		204.3***	
intra-nDNA $F_{d.f.=4}$	3.353**		2.120	
interaction $F_{d.f.=4}$	3.731***		5.530***	

** $p < 0.01$; *** $p < 0.001$

of *D*. *pseudoobscura* mtDNA under a nuclear background of the same species, but no remarkable difference in the case of *D*. *persimilis* (Hutter and Rand 1995). This discrepancy might be due uncontrolled differences in the nuclear genome.

We designed a cross scheme and generated coupled strains to homogenize the nuclear genome and show the effect of mitochondria if it exists. By repeated inbreeding for 10 generations with only 3 flies per generation, we generated coupled strains with a homogenized *D*. *albomicans* nuclear genome for 1 set and that of *D*. *nasuta* for the other. One male was shared by the coupled strains during that 10-generation period. Each set of coupled strains contained nearly the same nuclear background but different mitochondrial genomes. A set of coupled "A,a" strains had a homogenized nuclear composition from *D*. *albomicans*; the former had mitochondria from the same species while the latter had mitochondria from the other species, *D*. *nasuta*. Similarly, sets of coupled "N,n" strains were established. They were all highly inbred through repeated sib-mating. Since their establishment, those coupled strains have been cultured by non-overlapping generations. We established 5 sets of strains for each nuclear background to confirm that there was

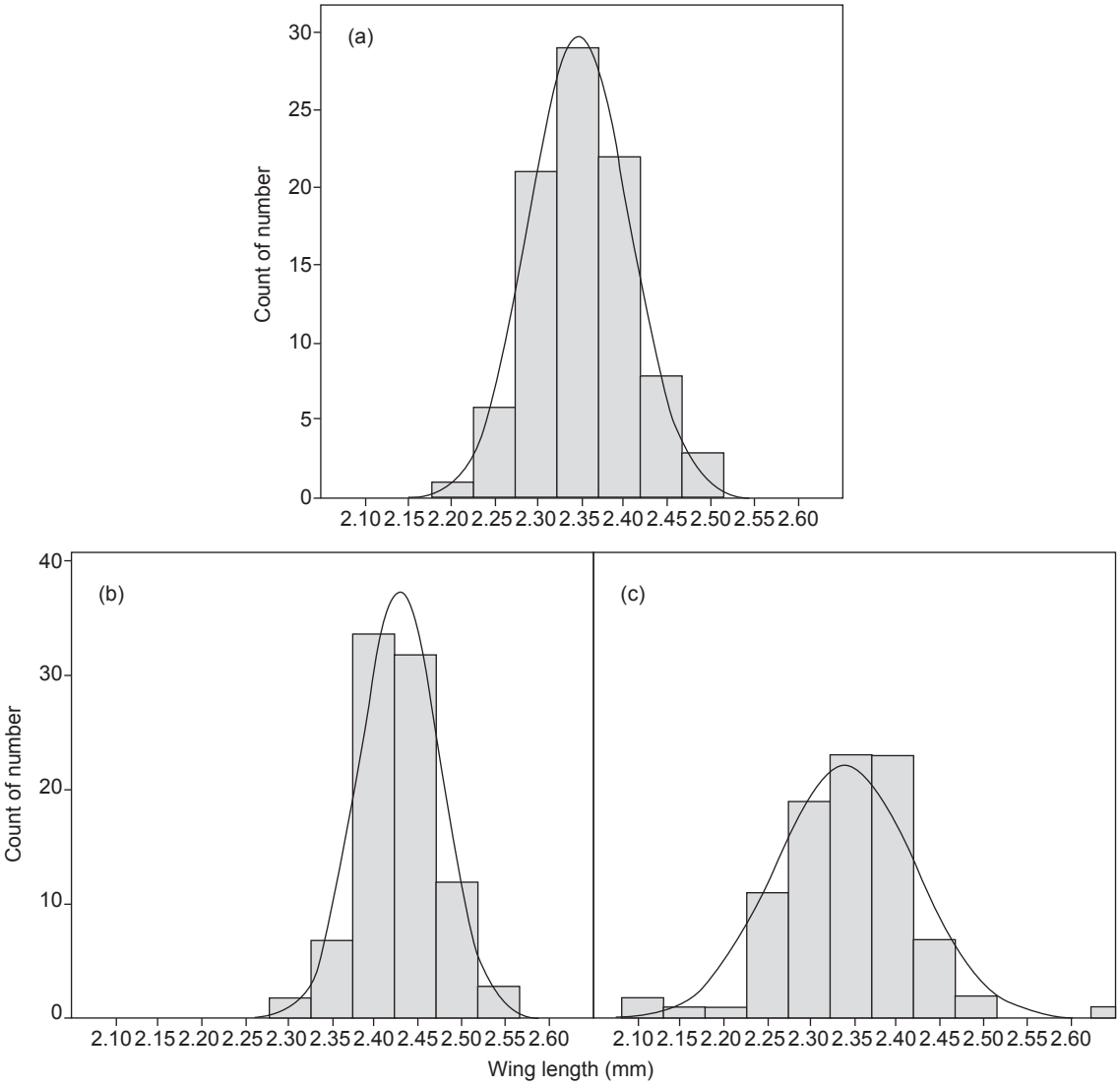

Fig. 5. Distributions of hybrid males are shown in histograms with the calculated normal distribution curves. (a) Ha_1, progeny of a *Drosophila nasuta* female crossed to *D*. *albomicans*. (b) AHa_2, progeny of Ha_1 males backcrossed to *D*. *albomicans*. (c) NHa_2, progeny of Ha_1 males backcrossed to *D*. *nasuta*.

no bias caused by selection or drift. These well-controlled materials are especially important for evaluating quantitative traits, such as reproductive ability and body size.

In analyzing the reproductive ability, coupled strains within the same set showed no difference, but strains with different nDNA differed significantly; for instance, "A,a" strains were more similar to *D*. *albomicans*, while "N,n" strains were closer to *D*. *nasuta*. Particularly evident in reproductive ability, strains with the *D*. *nasuta* nuclear genome had almost double the number of offspring than did strains with the *D*. *albomicans* nuclear genome regardless of which type of mtDNA they carried. As for the analysis of body size, the results of males and females were consistent. With a *D*. *albomicans* nuclear background, the interaction between mtDNA and nDNA was significant, while with a *D*. *nasuta* nuclear background, the influence of intraspecific nDNA was significant. None of them was influenced by introgressed mtDNA. In conclusion, no indication of mitochondrion-nuclear incompatibility between these 2 species was found, even thought they have coevolved for hundreds of thousand years after their divergence.

Chromosome interactions in this species pair are more complex than for other species because instead of the original sex chromosome arms, new sex chromosome arms exist. It has been demonstrated that X-autosome and Y-autosome interactions, rather than X-Y interactions, cause sterility (Johnson et al. 1992). However, considering the chromosome sets of Ha_1, NHa_2, and AHa_2 males respectively, Ha_1 males were homogeneous but NHa_2 and AHa_2 males were heterogeneous. Half of the NHa_2 males had exactly the same karyotype as the Ha_1 males, but instead of having one 2nd autosome from *D*. *albomicans* and 1 from *D*. *nasuta*, the other 1/2 had both 2nd autosomes from *D*. *nasuta*. AHa_2 males differed from Ha_1 males by having the sex chromosomes restored to the *D*. *albomicans* type. The autosome compositions of AHa_2 males were also of 2 kinds: one was the same as that of Ha_1 males and the other had both 2nd autosomes from *D*. *albomicans*. If the interaction between 2nd autosomes and sex chromosome had a major effect, the mean body size of NHa_2 would have deviated from that of Ha_1 for the composition change, but it did not. Moreover, if the interactions of 2nd autosomes and sex chromosomes were influential, the variance of AHa_2 males should have increased instead of decreasing when compared to Ha_1 males. X-autosome and Y-autosome interactions may be important in causing sterility, but they have not evolved in this species pair.

The 3rd autosome fused with the Y chromosome had a very different fate from the 3rd autosome fused with the X chromosome during the chromosomal evolution of *D*. *albomicans*. The new arm of the neo-Y in *D*. *albomicans* can only exist in males and therefore lacks recombination (Morgan 1912 1914). After coevolution of the neo-X for many generations, the neo-Y depends on the neo-X, but not vice versa. According to research on *D*. *miranda* (Bachtrog and Charlesworth 2002), neo-Y chromosomes may degenerate or lose some functions. Neo-X chromosomes can perform recombination in females, and may experience higher selection pressure in males if the homologous neo-Y degenerates. The neo-X can cooperate with the ancestral 3rd autosome and Y in Hn_1 males, but the neo-Y, which depends on its own neo-X, cannot perform well with the 3rd autosome and X in Ha_1 males. If this is an explanation for the smallest body size of Ha_1 males, it can be verified by backcross experiments. We expected to observe a similar situation, i.e., the same small size of male offspring from a backcross to *D*. *nasuta*, whereas the size of male offspring produced by a backcross to *D*. *albomicans* should revert because the neo-X can function well with the neo-Y. Our results showed that the body size of AHa_2 males reverted to the size of *D*. *albomicans*, while that of AHa_2 males remained the same. On the other hand, the autosomal composition was homogeneous in Ha_1, but it was variable in NHa_2, and AHa_2. Although no crossing over occurs in males of *Drosophila*, independent assortment generated a recombination between the 2nd and 4th autosomes. That might be the reason why the mean body size of NHa_2 was the same as that of Ha_1 but the variation increased from Ha_1 to NHa_2. These quantitative genetic influences of autosomes were observed as expected while abnormally smaller body size was observed when the sex chromosomes were incompatible. The decreased variation in concert with the increased mean from Ha_1 to AHa_2 supports cooperation between the neo-X and neo-Y being required to maintain a normal body size in our experimental system in which noise from autosomes was minimized. This evidence supports XY cooption.

Haldane's rule states that if only 1 hybrid sex is sterile or unviable, it is usually the heterozygous sex (Haldane 1922). Three explanations, including the dominance theory, the "faster-male" theory, and the "faster-X" theory, for Haldane's rule were

previously discussed in detail (Wu et al. 1996, Orr 1997). Haldane's rule represents an early stage in the evolution of postzygotic isolation, and inviability or sterility might arise very fast in males but much later in females (Coyne and Orr 1989 1997). However, the smaller hybrid males may have been a result of defects during development, which may occur at a very early stage of speciation even before sterility or inviability can be observed. Whether the neo-Y chromosome depends on being accompanied by the neo-X chromosome in hybrids as an initiation event for the appearance of sterility or inviability in the heterogametic sex warrants further investigation.

Acknowledgments: We thank Ms. Shu-Ping Huang for a help with the statisitical analysis and Mr. Eric Ott for suggestions that improved the original manuscript. This work was supported by grants (NSC86-2321-B002-069 and NSC87-2313-B002-052) from the National Science Council of Taiwan.

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