

## RESEARCH ARTICLES

# Genetic Exchange versus Genetic Differentiation in a Medium-Sized Inversion of *Drosophila*: The $A_2/A_{st}$ Arrangements of *Drosophila subobscura*

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Chromosomal inversion polymorphism affects nucleotide variation at loci associated with inversions. In *Drosophila subobscura*, a species with a rich chromosomal inversion polymorphism and the largest recombinational map so far reported in the *Drosophila* genus, extensive genetic structure of nucleotide variation was detected in the segment affected by the  $O_3$  inversion, a moderately sized inversion at Muller's element E. Indeed, a strong genetic differentiation all over  $O_3$  and no evidence of a higher genetic exchange in the center of the inversion than at breakpoints were detected. In order to ascertain, whether other polymorphic and differently sized inversions of *D. subobscura* also exhibited a strong genetic structure, nucleotide variation in 5 gene regions (P236, P275, P150, *Sxl*, and P125) located along the  $A_2$  inversion was analyzed in  $A_{st}$  and  $A_2$  chromosomes of *D. subobscura*.  $A_2$  is a medium-sized inversion at Muller's element A and forms a single inversion loop in heterokaryotypes. The lower level of variation in  $A_2$  relative to  $A_{st}$  and the significant excess of low-frequency variants at polymorphic sites indicate that nucleotide variation at  $A_2$  is not at mutation–drift equilibrium. The closest region to an inversion breakpoint, P236, exhibits the highest level of genetic differentiation ( $F_{ST}$ ) and of linkage disequilibrium (LD) between arrangements and variants at nucleotide polymorphic sites. The remaining 4 regions show a higher level of genetic exchange between  $A_2$  and  $A_{st}$  chromosomes than P236, as revealed by  $F_{ST}$  and LD estimates. However, significant genetic differentiation between the  $A_{st}$  and  $A_2$  arrangements was detected not only at P236 but also in the other 4 regions separated from the nearest breakpoint by 1.2–2.9 Mb. Therefore, the extent of genetic exchange between arrangements has not been high enough to homogenize nucleotide variation in the center of the  $A_2$  inversion.  $A_2$  can be considered a typical successful inversion of *D. subobscura* according to its relative length. Chromosomal inversion polymorphism of *D. subobscura* might thus cause the genome of this species to be highly structured and to harbor different gene pools that might contribute to maintain adaptations to particular environments.

## Introduction

Sturtevant (1917) was the first to consider the presence of inverted chromosome segments as naturally occurring genetic variation in *Drosophila*. Since then, polymorphism for paracentric inversions has been described in natural populations of most *Drosophila* species. In a study including 182 species, only 46 were monomorphic for chromosomal arrangements (Sperlich and Pfriem 1986). The detection of geographical clines and temporal cycles in the frequency of polymorphic inversions support that chromosomal inversion polymorphism is adaptive. In addition, some meiotic drive systems are associated with polymorphic inversions. Therefore, the study of chromosomal inversion polymorphism provides an exceptional opportunity to look for evidence of natural selection (reviewed in Krimbas and Powell 1992; Powell 1997).

Inversions have been shown to have several effects on recombination rates (reviewed in Roberts 1976). First, inversions relocate genes along a chromosome potentially modifying their recombinational context. Second, recombination is reduced in heterokaryotypes by the inhibition of chiasmata mainly near the breakpoints of paracentric inversions (Novitski and Braver 1954; Grell 1962). Finally, recombination is also reduced in heterokaryotypes, as single crossing over events within the inverted region give rise to

nonviable unbalanced meiotic products. The main consequence of a reduced recombination along inversions is to subdivide the population into 2 classes of chromosomes: standard and inverted. Those loci included in polymorphic inversions thus exhibit strong linkage disequilibrium (LD) in the population. Inversions could thus represent sets of coadapted gene complexes maintained by selection, as proposed by Dobzhansky (1970). Alternatively, a local adaptation mechanism that does not require coadaptation could also explain the maintenance of chromosomal inversion polymorphism (Kirkpatrick and Barton 2006). Standard and inverted chromosomes, however, are not completely isolated. Genetic exchange between chromosomal arrangements is possible as viable recombinant gametes arise by multiple crossing over events in the inverted region (reviewed in Ashburner 1989) and by gene conversion (Chovnick 1973). Through time, double crossover events will break down the associations between loci centrally located in the inversion. However, strong LD is still expected around the breakpoints and in regions where selection would maintain coadapted gene complexes (Navarro et al. 1997).

Inversions also affect nucleotide variation at loci associated with the inverted chromosomal region. The effect of inversions on nucleotide variation depends on the age of inversions and the rate of genetic exchange between inverted and standard chromosomes. Inversions reduce overall genetic variability as they quickly spread in a population. Indeed, the rapid increase in frequency of a new inversion causes a partial selective sweep that results in a depletion of nucleotide variation in inverted chromosomes. Thereafter, variation in the newly established inversion may be

**Key words:** inversion polymorphism, *Drosophila subobscura*, nucleotide polymorphism, genetic differentiation, genetic exchange,  $A_2$  and  $A_{st}$  arrangements.

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introduced by mutation and by genetic exchange with the ancestral arrangement either by double crossover events or by gene conversion. Gene conversion is expected to be uniformly distributed along the inversion except very close to the inversion breakpoints where it would be negligible. In contrast, genetic exchange by double crossover would be higher in the central part of the inversion (Navarro et al. 1997). Navarro et al. (2000) showed by computer simulations that a long time period is required for variation at inverted and standard chromosomes to attain mutation–drift–flux equilibrium. Moreover, they observed that once equilibrium had been attained, inversions can increase overall genetic variability around breakpoints due to the independent accumulation of mutations in the different arrangements and the lack of genetic exchange between them.

Empirical data partly support these theoretical predictions. First, a level of nucleotide variation much lower in inverted than in standard chromosomes has been detected not only at the breakpoints of some polymorphic inversions (Wesley and Eanes 1994; Andolfatto et al. 1999; Cáceres et al. 2001; Casals et al. 2003; Matzkin et al. 2005) but also at loci located near the breakpoints (Babcock and Anderson 1996). Second, a differential genetic exchange between inverted and standard chromosomes with a higher genetic differentiation at the breakpoints than in the center of the inversion has also been reported in polymorphic inversions such as the In(3L)Payne inversion of *Drosophila melanogaster* (Hasson and Eanes 1996) and the 2j inversion of *Drosophila buzzatii* (Laayouni et al. 2003). However, more complex patterns have also been detected (Mousset et al. 2003; Schaeffer et al. 2003; Schaeffer and Anderson 2005; Kennington et al. 2006). Finally, a rapid increase of overall nucleotide variability away from the breakpoints of different *D. melanogaster* polymorphic inversions has been described, suggesting that the studied inversions are not ancient balanced polymorphisms (Andolfatto et al. 2001).

In contrast to the previous studies, a rather homogeneous distribution of genetic exchange was detected along the  $O_3$  inversion in the  $O_{st}/O_3 + 4$  chromosomal system of *Drosophila subobscura* (Munté et al. 2005). Indeed, a strong genetic differentiation between inverted and non-inverted chromosomes prevailed all over  $O_3$  and the level of LD between chromosomal arrangements and the variants present at nucleotide polymorphic sites was quite uniformly distributed along the inversion. Thus, no evidence of double crossover inside the inversion was detected. Double crossover events are expected in inversions larger than 20 cM (Navarro et al. 1997). The recombinational length of the  $O_3$  inversion is 27.4 cM and thus, although  $O_3$  is a moderately small inversion, it seems to be large enough to support double crossover events. The complex double inversion loop present in  $O_{st}/O_3 + 4$  heterokaryotypes might have also contributed to prevent double crossover events. Alternatively, it could be argued that selection had acted against the recombinant chromosomes produced by double crossover events inside the inversion. The adaptive character of chromosomal inversion polymorphism in *D. subobscura* is well established (Prevosti et al. 1988), mainly in the  $O_{st}$  and  $O_3 + 4$  chromosomal arrangements (García and Prevosti 1981; Fontdevila et al. 1983). The pattern of nucleotide variation detected in the  $O_{st}/O_3 + 4$  chromosome system, with

a strong structuring that extends to ~4 Mb, is thus noteworthy, especially taking into account that the length of the recombinational map of *D. subobscura* (1007.6 cM) is the highest so far described in any *Drosophila* species (Cáceres et al. 1999). To assess whether this pattern can be extended to other small- or medium-sized inversions forming single or complex inversion loops is relevant to understand the role of chromosomal inversions in shaping and structuring nucleotide variation. Indeed, the genome of a species with a rich inversion polymorphism could be a mosaic of different gene pools that contribute to maintain adaptations to particular environments.

Here, we analyzed the level and pattern of nucleotide variation in 5 genome regions distributed along the  $A_2$  inversion in the  $A_{st}/A_2$  chromosome system of *D. subobscura*. The selected regions differ in their physical distance to the nearest breakpoint of the  $A_2$  inversion. This inversion has an estimated length of 41.3 cM and forms a single inversion loop in  $A_{st}/A_2$  heterokaryotypes. The obtained results show a significant genetic differentiation between the  $A_{st}$  and  $A_2$  arrangements all over the inversion, although there is evidence of a higher genetic exchange in the center of the inversion than near breakpoints. Therefore, even for this medium-sized inversion, the genetic exchange between arrangements has not been high enough to homogenize nucleotide variation in the central part of the inversion.

## Materials and Methods

### Genomic Regions Studied

Four regions (P236, P150, P125, and P275) were selected after the mapping by in situ hybridization of several recombinant phages randomly isolated from a genome library of *D. subobscura* (Papaceit et al. 2006). The fifth region corresponded to a fragment of the *Sex-lethal* (*Sxl*) gene including part of exons 4 and 5 and the intron between them. *Sxl* was cloned and sequenced in *D. subobscura* by Penalva et al. (1996), and the sequence is available in the EMBL database with GenBank accession number X98370. The location relative to the  $A_2$  inversion of the 5 studied regions is shown in figure 1. The breakpoints of the  $A_2$  inversion are 8C/D–12C/D (Krimbas 1992). The recombination length of the  $A_2$  inversion was inferred considering a total length of 150 cM for the A chromosome of *D. subobscura* (Spurway 1945). The distance between a region and the nearest breakpoint was estimated assuming that the euchromatic portion of the *D. subobscura* A chromosome has 25.8 Mb (Richards et al. 2005) that are homogeneously distributed.

### Isolation of Genomic Regions

The DNA from the selected recombinant phages was purified using the QIAGEN (Chatsworth, CA) kit following manufacturer's instructions. Phage DNA was digested with the suitable restriction enzymes to release phage arms, cloned into pBluescript SK<sup>+</sup>, and used to transform XL1-Blue *Escherichia coli* competent cells (Stratagene, La Jolla, CA). Insert sizes of the recombinant plasmids were screened by polymerase chain reaction (PCR) (Kilger and Schmid 1994). Plasmid DNA was purified, and both ends of

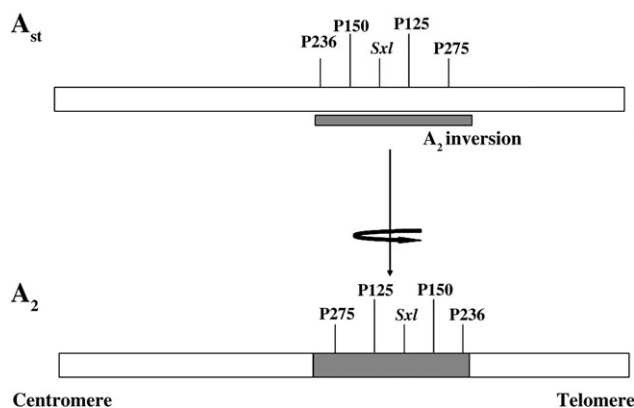


FIG. 1.—Location of the 5 studied regions along the A<sub>st</sub> (top) and the A<sub>2</sub> (bottom) arrangements of *Drosophila subobscura*.

each insert were sequenced using the SK and T7 universal primers. After Blast searches on the complete genomes of *D. melanogaster* and *Drosophila pseudoobscura*, regions of about 2 kb from each phage were selected for further analysis. The selected fragments were completely sequenced by primer walking.

### Fly Samples

A natural population of *D. subobscura* from the Madeira Island was sampled in 2001. Highly inbred lines were established after at least 12 generations of sib mating. Males of each inbred line were crossed with virgin females of the *chcu* line that is homozygous for the A<sub>st</sub> arrangement. Salivary glands from third-instar larvae of these crosses were dissected, stained, and squashed according to the standard protocol. The chromosomal arrangement of each line was determined by the observation of polytene chromosomes under a microscope. Twelve A<sub>2</sub> lines and 6 A<sub>st</sub> lines were selected for further analysis. The *chcu* strain of *D. subobscura* was also used to obtain a reference sequence. A highly inbred line of *Drosophila madeirensis* was used for interspecific comparisons.

### DNA Sequencing

Genomic DNA was extracted using a modification of protocol 48 in Ashburner (1990), and the selected regions were subsequently PCR amplified. Sequencing reactions were carried out with the Big Dyes 3.1 cycle sequencing kit. Samples were run in the ABI Prism 3700 automated sequencer (Applied Biosystems, Foster City, CA). Both strands of the purified PCR products were sequenced. Partial sequences were assembled with the SeqMan program, and complete sequences were multiply aligned with Megalign. These programs are included in the DNASTAR LaserGene v 6.0 software package. Further edition of sequences was performed with the MacClade v 4.05 program (Maddison DR and Maddison WP 2002).

### Data Analysis

Analyses were performed for each region independently and for a single concatenated data set including

all regions. Standard parameters of nucleotide polymorphism were estimated: the number of segregating sites (*S*), the minimum number of mutations ( $\eta$ ), nucleotide diversity ( $\pi$ ; Nei 1987), and heterozygosity per site ( $\theta$ ; Watterson 1975). The nucleotide divergence per silent site ( $K_{sil}$ ) between *D. subobscura* and *D. madeirensis* was inferred according to Nei and Gojobori (1986). The level of genetic differentiation between arrangements was estimated as  $D_{XY}$  (Nei 1987),  $D_a$  (Nei 1987), and  $F_{ST}$  (Hudson et al. 1992) and its significance established using the  $K_s^*$  statistic and the permutation test (Hudson et al. 1992). Gene conversion tracts were detected by the algorithm proposed by Betrán et al. (1997). The probability that the observed number of polymorphisms shared between arrangements could be explained by recurrent mutation was inferred according to the hypergeometric distribution as described in Rozas and Aguadé (1994).

The LD between pairs of parsimony informative sites and association between informative sites and chromosomal arrangement was estimated by the  $r^2$  statistic (Hill and Robertson 1968) and its statistical significance assessed by the  $\chi^2$  test with the Bonferroni correction for multiple comparisons (Weir 1996).

Neutrality tests (Hudson et al. 1987; Tajima 1989; Fu and Li 1993) were performed separately for each chromosomal arrangement. Statistical significance for all tests was assessed by coalescent simulations (10,000 replicates) conditioned on *S* under the conservative assumption of no intragenic recombination. *Drosophila madeirensis* was used as the outgroup species in those tests that required interspecific data. The DnaSP v 4.1 program (Rozas et al. 2003) was used to perform most of the analyses and the HKA program (Hey 2004) for the neutrality multilocus tests.

Gene genealogies were reconstructed by the Neighbor-Joining method (Saitou and Nei 1987) as implemented in the MEGA 3 program (Kumar et al. 2004) and using the number of substitutions per site corrected according to the Kimura (1980) 2-parameter method as the genetic distance.

## Results

### Nucleotide Polymorphism

The multiple alignment of the 5 studied regions in the concatenated data set including the 18 *D. subobscura* lines had 6,857 sites after excluding alignment gaps. A total of 230 polymorphic sites (113 singletons) were detected, and the minimum number of mutations was 232 (9 in coding and 212 in noncoding regions). A detailed description of nucleotide polymorphism is shown in supplementary figures 1–5 (Supplementary Material online).

### Gene Flow and Genetic Differentiation between Arrangements

Significant genetic differentiation between the A<sub>st</sub> and the A<sub>2</sub> arrangements was detected in each of the 5 studied regions as well as in the concatenated data set (table 1). Therefore, both arrangements were genetically differentiated despite the lack of fixed differences between them and the presence of 60 shared polymorphisms. The large

**Table 1**  
Genetic Differentiation between the A<sub>2</sub> and A<sub>st</sub> Chromosomal Arrangements

	Shared <sup>a</sup>	Fixed <sup>b</sup>	$S_{x1}$ <sup>c</sup>	$S_{x2}$ <sup>d</sup>	$D_{XY}$ <sup>e</sup>	$D_a$ <sup>f</sup> (standard error)	$F_{ST}$ <sup>g</sup>	$P(K_s^*)$ <sup>h</sup>
P236	15	0	22	13	0.0129	0.0060 (0.0028)	0.464	0.001**
P150	7	0	22	15	0.0072	0.0008 (0.0012)	0.145	0.003**
<i>Sxl</i>	11	0	17	11	0.0055	0.0005 (0.0011)	0.096	0.020*
P125	24	0	34	8	0.0128	0.0021 (0.0024)	0.156	0.030*
P275	5	0	20	13	0.0083	0.0017 (0.0017)	0.210	0.002**
Concatenated data	60	0	114	58	0.0092	0.0023 (0.0016)	0.249	0.000***

<sup>a</sup> Polymorphic sites segregating for the same 2 variants in both arrangements.

<sup>b</sup> Fixed differences between arrangements.

<sup>c</sup> Sites polymorphic in A<sub>2</sub> and monomorphic in A<sub>st</sub>.

<sup>d</sup> Sites polymorphic in A<sub>st</sub> and monomorphic in A<sub>2</sub>.

<sup>e</sup> Average number of nucleotide differences per site between arrangements corrected according to Jukes and Cantor (1969).

<sup>f</sup> Net number of nucleotide differences per site between arrangements corrected according to Jukes and Cantor (1969). The standard error is indicated in brackets.

<sup>g</sup> Proportion of nucleotide diversity attributable to variation among arrangements.

<sup>h</sup> Probability of the permutation test based on the  $K_s^*$  statistic.

\*0.01 <  $P$  < 0.05; \*\*0.001 <  $P$  < 0.01; \*\*\* $P$  < 0.001.

number of shared polymorphisms cannot be explained by recurrent mutation according to the hypergeometric distribution. Therefore, it has to be inferred that genetic exchange between the 2 arrangements contributed to the presence of shared polymorphisms. Indeed, some gene conversion tracts were detected in P236, P125, and P275. The genetic differentiation between arrangements was also analyzed in relation to the distance of each region to the A<sub>2</sub> inversion nearest breakpoint (fig. 2). The highest genetic differentiation was detected at P236 that is the nearest region to a breakpoint. However, genetic differentiation is significant even in the central part of the inversion loop. No clear relationship between distance and genetic differentiation was detected among the P150, *Sxl*, P125, and P275 regions.

### Nucleotide Variation

Nucleotide diversity and heterozygosity per site were estimated independently for each chromosomal arrangement due to the detected genetic differentiation between them. Nucleotide diversity estimates (either  $\pi_{total}$  or  $\pi_{sil}$ ) were slightly higher in A<sub>st</sub> than in A<sub>2</sub> in the concatenated data set and in each of the 5 studied regions except P125 (table 2). Silent nucleotide diversity in each region was divided by silent divergence between *D. subobscura* and *D. madeirensis* to account for differences in the mutation rate among regions. The corrected estimates were analyzed in relation to the distance of each region to the nearest breakpoint (fig. 3). The nearest region to an inversion breakpoint (P236) showed the lowest level of variation in both arrangements. However, the low level of variation relative to divergence at P236 was not significant. None of the HKA tests (Hudson et al. 1987) performed between P236 and the other studied regions was significant either in A<sub>2</sub> or A<sub>st</sub>. A similar result was obtained in pairwise comparisons between P150, *Sxl*, P125, and P275 regions. The HKA multilocus tests performed within arrangement were also not significant ( $\chi^2 = 1.65$ , 8 degrees of freedom [df],  $P = 0.98$  in A<sub>2</sub> and  $\chi^2 = 1.25$ , 8 df,  $P = 0.99$  in A<sub>st</sub>). Therefore, no significant heterogeneity in the level of polymorphism relative to divergence among the different regions was detected.

The HKA test was also used to contrast whether the level of polymorphism relative to divergence detected in

the 5 X-linked gene regions here studied was similar to that previously reported in other autosomic regions of *D. subobscura*. The test was applied using the concatenated data for the A chromosome regions (either A<sub>st</sub> or A<sub>2</sub>) and the concatenated data for the O chromosome regions (either O<sub>st</sub> or O<sub>3+4</sub>) in Munté et al. (2005). None of the performed HKA tests was significant, which indicates that the ratio polymorphism/divergence does not differ significantly among these regions included in different chromosome inversions.

### LD Analysis

The association between chromosomal arrangement and the variants at informative polymorphic sites (measured as the average  $r^2$ ) was analyzed in each region in relation to its distance to the nearest inversion breakpoint (fig. 4). Nucleotide variation at P236 (the nearest region to an inversion breakpoint) showed the strongest association with arrangements. The extent of association was quite similar in the other 4 regions.

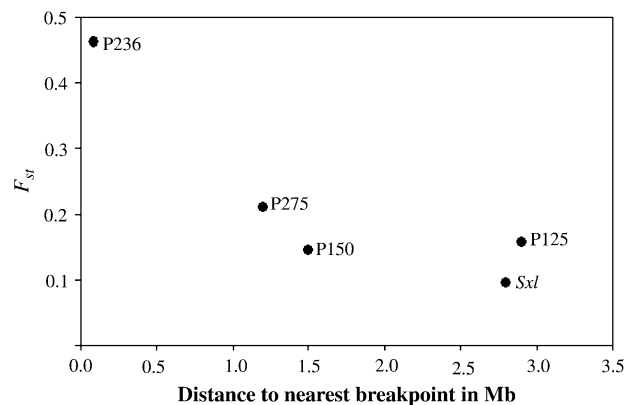


FIG. 2.—Genetic differentiation between arrangements in each studied region versus its distance to the nearest breakpoint of the A<sub>2</sub> inversion. The distance between each region and the nearest inversion breakpoint was estimated assuming a homogenous DNA distribution along the A<sub>2</sub> inversion (7.1 Mb).

**Table 2**  
Nucleotide Variation and Divergence within Chromosomal Arrangement

		$n^a$	$m^b$	$S^c$	$\pi_{\text{total}}^d$	$\pi_{\text{sil}}^e$	$\theta_{\text{sil}}^f$	$K_{\text{sil}}^g$
P236	A <sub>2</sub>	12	1,531	37 (18)	0.0060	0.0060	0.0080	0.026
	A <sub>st</sub>	6	1,531	28 (18)	0.0076	0.0076	0.0080	0.024
P150	A <sub>2</sub>	12	1,262	26 (17)	0.0050	0.0054	0.0074	0.011
	A <sub>st</sub>	6	1,262	18 (14)	0.0053	0.0063	0.0072	0.012
Sxl	A <sub>2</sub>	12	1,630	28 (18)	0.0042	0.0047	0.0063	0.015
	A <sub>st</sub>	6	1,630	21 (11)	0.0056	0.0063	0.0065	0.016
P125	A <sub>2</sub>	12	1,460	57 (29)	0.0109	0.0109	0.0129	0.023
	A <sub>st</sub>	6	1,460	31 (10)	0.0105	0.0105	0.0093	0.024
P275	A <sub>2</sub>	12	974	25 (15)	0.0061	0.0061	0.0085	0.019
	A <sub>st</sub>	6	974	18 (15)	0.0069	0.0069	0.0080	0.017
Concatenated data	A <sub>2</sub>	12	6,857	173 (97)	0.0065	0.0067	0.0085	0.019
	A <sub>st</sub>	6	6,857	116 (68)	0.0072	0.0079	0.0082	0.019

<sup>a</sup> Sample size.

<sup>b</sup> Number of sites after excluding sites with alignment gaps.

<sup>c</sup> Number of polymorphic sites. The number of singletons is shown in brackets.

<sup>d</sup> Nucleotide diversity in all sites.

<sup>e</sup> Silent nucleotide diversity.

<sup>f</sup> Silent heterozygosity per site based on the number of segregating sites.

<sup>g</sup> Silent divergence per site between *Drosophila subobscura* and *Drosophila madeirensis*.

The association between informative polymorphic nucleotide sites was also analyzed. In the concatenated data set including all sequences (A<sub>2</sub> + A<sub>st</sub>) with 115 informative sites, a total of 18.4% of pairwise comparisons showed significant LD by the  $\chi^2$  test ( $P < 0.05$ ). This value dropped to 11.8% and 10.6% within A<sub>2</sub> and A<sub>st</sub>, respectively. A similar result was obtained when only interlocus associations were considered (i.e., LD between informative sites from different regions). The percentage of significant interlocus comparisons was 15.4% in the total sample but 9.6% and 1.1% in A<sub>2</sub> and A<sub>st</sub>, respectively. Therefore, the presence of both arrangements not only contributes to increase overall LD but also to maintain LD between the loci included in the A<sub>2</sub> inversion.

### Patterns of Polymorphism

Tajima's  $D$  (1989) and Fu and Li's  $D$  (1993) statistics were negative in the 5 studied regions in A<sub>2</sub> and in 4 of them in A<sub>st</sub> (table 3). However, none of the performed tests was statistically significant. These data were also analyzed by a multilocus test based on the average value of the Tajima's test statistic ( $\bar{D}$ ). In A<sub>st</sub>, the empirical  $D$  value averaged across the 5 studied regions ( $\bar{D} = -0.3248$ ) was not significantly lower than the average  $D$  value obtained from the computer simulations ( $P = 0.25$ ). However, the multilocus test was statistically significant for the A<sub>2</sub> arrangement ( $\bar{D} = -1.0824$ ;  $P = 0.005$ ). This result indicated a significant excess of low-frequency variants, mostly singletons, in this arrangement. A similar result was obtained for the multilocus test based on Fu and Li's  $D$  statistic:  $\bar{D} = -0.5267$ ,  $P = 0.16$  in A<sub>st</sub> and  $\bar{D} = -1.2962$ ,  $P = 0.004$  in A<sub>2</sub>.

### Gene Genealogy

The gene genealogy reconstructed from total variation in the concatenated data set is shown in figure 5. There was a partial clustering of lines according to their chromosomal arrangement. Most A<sub>2</sub> lines grouped together in a single cluster with a 96% bootstrap support after 1,000 replicates.

However, 2 A<sub>2</sub> lines clustered with the A<sub>st</sub> lines. These lines present gene conversion tracts in 3 of the 5 studied regions and thus genetic exchange between both arrangements may explain the clustering of both A<sub>2</sub> lines with the A<sub>st</sub> lines. Indeed, when the sequences with gene conversion tracts were removed from the analysis, the *D. subobscura* lines group in 2 clusters according to their chromosome arrangement: the A<sub>st</sub> and the A<sub>2</sub> clusters with 100% and 99% support, respectively. The gene genealogy is thus consistent with a monophyletic origin of A<sub>2</sub>.

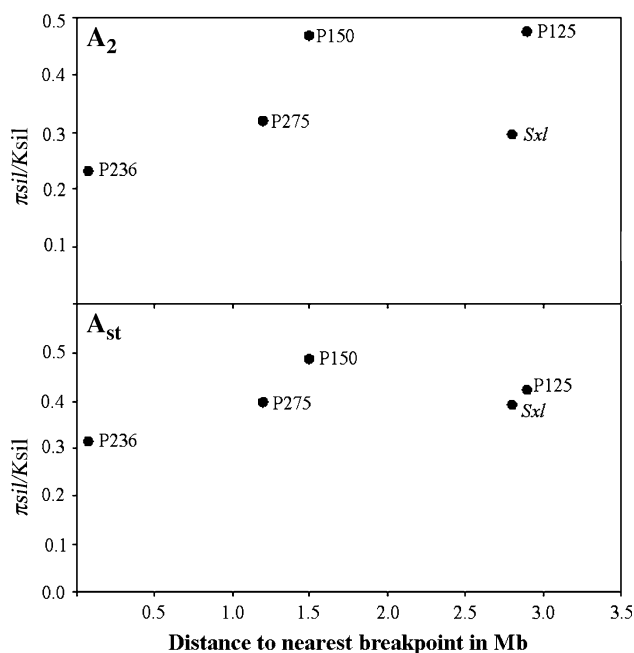


FIG. 3.—The  $\pi_{\text{sil}}/K_{\text{sil}}$  estimates in the A<sub>2</sub> (top) and A<sub>st</sub> (bottom) arrangements in each studied region versus its distance to the nearest breakpoint of the A<sub>2</sub> inversion. Silent divergence per site ( $K_{\text{sil}}$ ) was estimated between *Drosophila madeirensis* and each *Drosophila subobscura* arrangement. The distance to the nearest breakpoint was inferred as in figure 2.

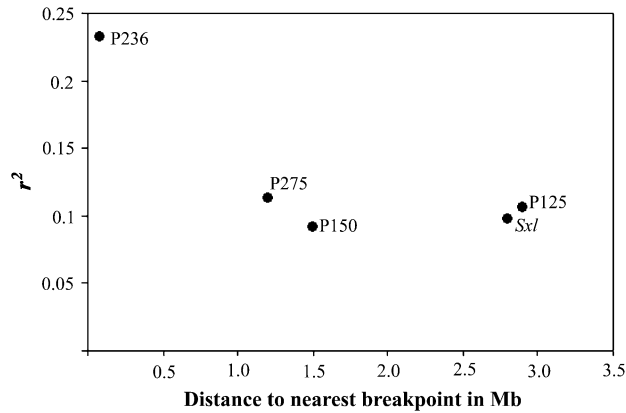


FIG. 4.—Association between chromosomal arrangements and variants at informative polymorphic sites in each region (average  $r^2$ ) versus its distance to the nearest inversion breakpoint. The distance to the nearest breakpoint was inferred as in figure 2.

#### Age of the $A_2$ Inversion

The age of the  $A_2$  inversion was estimated as proposed by Rozas et al. (1999) according to the expansion model (Rogers and Harpending 1992; Rogers 1995). If  $A_2$  is in the transient phase to mutation–drift equilibrium, the level of silent nucleotide diversity in  $A_2$  would be equal to  $2\mu t$ , where  $\mu$  is the neutral mutation rate and  $t$  the time since its origin. This approach assumes that all variation within the inversion has originated by mutation. For this reason, we estimated the age of the  $A_2$  inversion based only on silent nucleotide variation at the *Sxl* and P150 regions, the 2 gene regions where no gene conversion tracts were detected. The neutral mutation rate was inferred from the overall silent nucleotide divergence between *D. subobscura* and *D. madeirensis* (0.019; table 2) assuming a divergence time of 0.63 Myr for the 2 species (Ramos-Onsins et al. 1998):  $15 \times 10^{-9}$  substitutions per site per year. According to this approach, the estimated age of the  $A_2$  inversion would be 160,000 years.

**Table 3**  
Tajima's and Fu and Li's Tests of Neutrality within Chromosomal Arrangement

		Tajima's $D$	Fu and Li's $D$
P236	$A_2$	−1.1042	−0.5992
	$A_{st}$	−0.2729	−0.2924
P150	$A_2$	−1.1648	−1.8375
	$A_{st}$	−0.8545	−1.2853
<i>Sxl</i>	$A_2$	−1.1424	−1.7074
	$A_{st}$	−0.2397	−0.2860
P125	$A_2$	−0.7584	−0.8139
	$A_{st}$	0.5971	1.0081
P275	$A_2$	−1.2425	−1.5233
	$A_{st}$	−0.8545	−0.7811
Concatenated data	$A_2$	−1.0634	−0.9146
	$A_{st}$	−0.2185	−0.2264

NOTE.—*Drosophila madeirensis* was used as the outgroup species in the Fu and Li's  $D$  test.

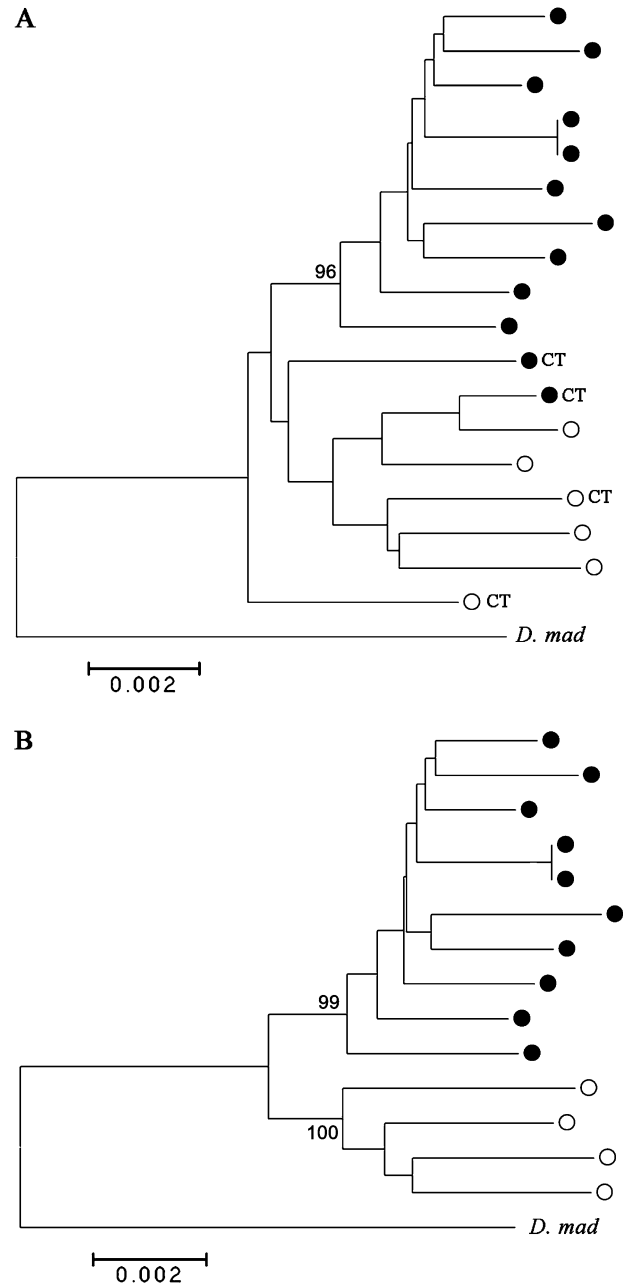


FIG. 5.—Neighbor-Joining gene genealogies based on total nucleotide variation in the concatenated data set. (A) Gene genealogy including all lines; CT indicates lines with gene conversion tracts. (B) Gene genealogy after excluding lines with gene conversion tracts. Numbers refer to bootstrap percentages higher than 95 for those clusters including lines with the same gene arrangement. Solid dots refer to the *Drosophila subobscura*  $A_2$  lines and open dots to  $A_{st}$ . *D. mad* indicates the *Drosophila madeirensis* line used as the outgroup.

#### Discussion

##### Nucleotide Diversity and Insularity

Levels of silent nucleotide diversity in the 5 genome regions of the *D. subobscura* population from the Madeira Island here studied range from 0.0042 to 0.0109 in  $A_2$  and from 0.0063 to 0.0105 in  $A_{st}$ . Overall diversity in the concatenated data is 0.0067 and 0.0079 in  $A_2$  and  $A_{st}$ ,

respectively. These estimates are similar to those reported in European populations at yellow in the  $A_{st}$  chromosomal class (0.008; Munté et al. 2000) and slightly lower than those present in the noncoding region of the *Rp11215* gene (0.011 in  $A_2$  and 0.014 in  $A_{st}$ ; Llopart and Aguadé 2000). In addition, the polymorphism to divergence ratio did not differ significantly, according to the HKA test results, between the A chromosome regions from Madeira and the O chromosome regions from Europe (Munté et al. 2005). Therefore, the insularity of the *D. subobscura* population here studied does not seem to affect the level of nucleotide variation, which is consistent with previous data for the autosomic *rp49* gene region (Khadem et al. 2001).

### The $A_{st}/A_2$ Chromosome System

The  $A_2$  inversion is widespread in the *D. subobscura* palearctic distribution area and reaches the highest frequencies in Spain and northeast Africa (Krimbas 1992).  $A_2$  seems to be fixed in Tenerife and has a frequency of about 90% in Madeira (Prevosti 1972; Larruga et al. 1983). The  $A_2$  arrangement has given rise to other more complex arrangements such as  $A_{2+6}$  (with frequencies higher than 50% in Africa) and  $A_{2+3+5+7}$  (the sex-ratio arrangement of *D. subobscura*). Chromosomal phylogenies suggested that  $A_{st}$  was most likely the ancestral arrangement from which the  $A_2$  arrangement originated. Indeed, the A chromosome arrangements of *D. madeirensis* and the close relative *Drosophila guanche* are derived from the standard arrangement in the chromosomal segment affected by the  $A_2$  inversion (Moltó et al. 1987; Papaceit and Prevosti 1991). The level and pattern of variation in  $A_2$  relative to  $A_{st}$  in the 5 genome regions here studied also support the derived character of  $A_2$ . Indeed, the selective sweep produced by the establishment of a new inversion would cause a strong reduction of nucleotide variation and an excess of low-frequency variants at loci associated with the inversion. The low level of variation in  $A_2$  relative to  $A_{st}$  and the highly significant Tajima's  $D$  and Fu and Li's  $D$  multilocus tests in  $A_2$ , with negative values for all tests statistics, strongly support that  $A_2$  is not at mutation-drift equilibrium but in the transient phase of recovering nucleotide variation after its origin. The estimate of the age of the  $A_2$  inversion is of about 160,000 years. The  $A_2$  inversion is thus substantially younger than the  $O_{st}$  and  $O_{3+4}$  arrangements of *D. subobscura* with an age of 0.24 and 0.33 Myr, respectively (Rozas et al. 1999), but slightly older than the  $A_1$  arrangement with an age of 126,000 years (Munté et al. 2000).

### Effect of the $A_{st}/A_2$ Chromosome System on Nucleotide Variation

The  $A_2/A_{st}$  chromosomal polymorphism affects nucleotide variation at the loci included in the  $A_2$  inversion. Although none of the HKA tests performed was significant, the lowest silent nucleotide diversity corrected by silent divergence, both in  $A_{st}$  and  $A_2$ , was detected at P236, the gene region closest to an inversion breakpoint (fig. 3). In addition, nucleotide variation along the  $A_2$  inversion is highly structured, and inverted and noninverted chromosomes are genetically differentiated even in the center of the inversion

loop. The strongest genetic differentiation is detected near the inversion breakpoint at P236. Indeed, P236 exhibits the highest  $F_{ST}$  estimate (fig. 2), the highest LD with chromosomal arrangement (fig. 4), and the lowest probability in the permutation test based on the  $K_s^*$  statistic (table 1). Despite the strong genetic differentiation, evidence of genetic exchange by gene conversion was detected at P236. Therefore, the distance of P236 to the breakpoint is high enough to allow gene conversion. The other 4 regions studied exhibit a higher level of genetic exchange (lower  $F_{ST}$  estimates) and a lower LD with chromosomal arrangements than P236. Likely, both gene conversion and double cross-over contribute to the genetic exchange in these regions that are more distant to the breakpoint than P236. The distance of these regions to the nearest breakpoint ranges from about 1.2 to 2.9 Mb, but these differences in distance do not seem to affect the level of genetic exchange between inverted and noninverted chromosomes. Gene conversion is expected to be uniform along an inversion, but double cross-overs are expected to fit a parabolic curve with the highest exchange in the center of the inversion (Navarro et al. 1997). Machado et al. (2007) proposed that, as an approximation, the decay of genetic differentiation when moving away from the inversion breakpoint might show a logarithmic form. The logarithmic regression is significant for both  $F_{ST}$  ( $n = 5$ ,  $r^2 = 0.959$ ,  $P = 0.0035$ ) and LD between informative polymorphic sites and arrangement ( $n = 5$ ,  $r^2 = 0.928$ ,  $P = 0.0084$ ). However, this result has to be taken with caution because the approximately 1.2-Mb region between the breakpoint and P275 is covered by a single marker. Additional data on markers covering this region would be required to assess the pattern of decay in genetic differentiation along the  $A_2$  inversion and most importantly to confirm the detected pattern of genetic differentiation and LD, which is now based on a single marker near the inversion breakpoint.

### The $A_{st}/A_2$ versus the $O_{3+4}/O_{st}$ Chromosome Systems

The  $O_3$  inversion has a recombinational length of about 27.4 cM, and in *D. subobscura*, it is always found with the overlapping inversion  $O_4$  forming the  $O_{3+4}$  arrangement. Thus,  $O_{3+4}/O_{st}$  heterokaryotypes present a complex double inversion loop. In contrast, the length of the  $A_2$  inversion is of about 41.3 cM and  $A_2$  forms a single inversion loop in  $A_2/A_{st}$  heterokaryotypes. These differential characteristics might affect the level of genetic differentiation along the  $O_3$  and  $A_2$  inversions, but similarities in the extent of differentiation in both inversions can also be detected.  $F_{ST}$  estimates between  $O_{3+4}$  and  $O_{st}$  chromosomes along the  $O_3$  inversion range from 0.43 to 0.66 (Munté et al. 2005). The  $F_{ST}$  estimate at P236 (the nearest region to an  $A_2$  inversion breakpoint) is 0.46 and thus within but close to the lower bound of this range. Estimates of genetic differentiation in the other regions are lower (from 0.21 to 0.096) than at P236. This result indicates that genetic exchange in the center of the inversion loop in  $A_2/A_{st}$  heterozygotes is higher than in the center of the loop formed by  $O_3$  in  $O_{3+4}/O_{st}$  heterozygotes. However, genetic differentiation between  $A_{st}$  and  $A_2$  chromosomes is still significant in the center of the  $A_2$  inversion and highly significant in the

concatenated data set. In addition, the presence of the  $A_2$  inversion contributes to maintain interlocus LD. Therefore, the genetic exchange along the  $A_2$  inversion has not been high enough to homogenize the gene content between  $A_{st}$  and  $A_2$  chromosomes in the inverted segment. Moreover, the inferred age of the  $A_2$  arrangement and its pattern of variation would indicate that the  $A_2/A_{st}$  system is not at mutation–drift–flux equilibrium.

$A_2$  is a medium-sized *Drosophila* inversion with a relative physical length of 4.7% (i.e., the physical length of the inversion relative to the total physical length of the species). The evolutionary success of an inversion partly depends on its length. An excess of medium sized inversions, either fixed or polymorphic, was detected in the *D. buzzatii* species complex (Cáceres et al. 1997). The recombinational length but not the physical length seems, however, to be the important factor determining the success of an inversion (Cáceres et al. 1999). Indeed, the physical length of successful inversions is inversely correlated to the species recombination map length (i.e., successful inversions tend to be shorter in species with larger recombination maps). The average physical length of the 28 polymorphic successful inversions in *D. subobscura* is  $5.4 \pm 1.62\%$  (Cáceres et al. 1999) and thus the relative physical length of  $A_2$  is within its confidence interval. Therefore, the genetic content of most successful polymorphic inversions in *D. subobscura* would be expected to be genetically differentiated between inverted and noninverted chromosomes. Moreover, genetic exchange between different arrangements might be further prevented because a large fraction of the successful polymorphic inversions form complex gene arrangements of overlapping inversions, such as  $J_{3+4}$ ,  $U_{1+2+8}$ ,  $E_{1+2+9+12}$ , and  $O_{3+4+8}$ .

In contrast to  $A_2$ , the  $O_3$  inversion can be considered a moderately small inversion (2.78% in physical length). This inversion (forming the  $O_{3+4}$  arrangement) turned out to maintain strong LD over large distances (~4 Mb). Indeed, genetic differentiation and LD between arrangements and polymorphic sites was quite uniform along the inversion. Therefore, the  $O_{3+4}/O_{st}$  chromosome system would be very efficient in keeping together coadapted gene complexes. Genetic exchange in the center of the medium sized  $A_2$  inversion is, however, higher than in  $O_3$ . Putative double crossover events inside the  $A_2$  inversion loop are thus more likely to break down putative coadapted gene complexes maintained by  $A_2$ . If selection acted against the recombinant chromosomes that break coadapted gene complexes, genes included in these complexes would be expected to present stronger LD than those not included in them. The presence of regions of high association interspersed by regions of low association inside an inversion has been proposed as a proof of coadaptation (Schaeffer et al. 2003; Kennington et al. 2006). In the present study, similar levels of LD with chromosomal arrangement were detected in gene regions 1.2–2.9 Mb apart from the  $A_2$  inversion breakpoints. Therefore, no evidence that the studied regions were included in coadapted gene complexes was found. A more dense coverage along the  $A_2$  inversion would be required to identify regions included in putative coadapted gene complexes maintained by the  $A_{st}/A_2$  arrangements of *D. subobscura*.

## Supplementary Material

Supplementary figures 1–5 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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