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Evolution

Transfer of genetic information in the *rp49* region of *Drosophila subobscura* between different chromosomal gene arrangements

(chromosomal polymorphism/nucleotide polymorphism/population genetics/molecular evolution/ribosomal protein)

JULIO ROZAS* AND MONTSERRAT AGUADÉ

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08071 Barcelona, Spain

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ABSTRACT **Nucleotide variation in the region including the ribosomal protein 49 (*rp49*) gene was investigated by direct sequencing of 10 alleles of *Drosophila subobscura* from chromosomes differing in gene arrangement. Fifty-six nucleotide and seven length polymorphisms were detected over a 1.5-kb region. Of the 20 nucleotide polymorphisms present more than once in the sample, 13 were segregating in $O_{[3+4]}$, 9 in $O_{[3+4+8]}$, and 4 in $O_{[st]}$ chromosomal classes. Several of these polymorphisms were segregating in more than one chromosomal class, a strong indication of genetic transfer between different chromosomal gene arrangements either by double crossover or gene conversion. Given the probable role played by gene conversion in the history of the *rp49* region in *D. subobscura*, estimates of nucleotide diversity within chromosomal class indicate that the $O_{[3+4]}$ chromosomal gene arrangement is older than the $O_{[st]}$ arrangement.**

Inversion polymorphism is widespread in different species of *Drosophila* and has been extensively studied in two noncosmopolitan species of the obscura group, *D. pseudoobscura* and *D. subobscura*, as well as in *D. melanogaster*. It is generally considered that inversions are monophyletic and that recombination is severely reduced in inversion heterozygotes. Different gene arrangements would therefore accumulate different mutations and their gene content would differentiate. Strong associations between several allozyme loci and inversions have been detected in the above-mentioned species. Although these associations were first interpreted as being adaptive (1, 2), they were later considered to be compatible with a neutral explanation, given the monophyletic origin of inversions and the low rate of recombination in inversion heterozygotes (3, 4). Discrimination between the selective and historical hypotheses (5) requires knowledge of the effective number of inverted and noninverted chromosomes, the rate of recombination (by double crossover or gene conversion), and the age of inversions. However, there are very few estimates of the rate of gene conversion (6, 7).

Analysis of restriction map variation in different genomic regions—the *Adh* region in *D. melanogaster* (8), the *rp49* region in *D. subobscura* (9), and the *Amy* region in *D. pseudoobscura* (10)—has revealed the presence of particular polymorphisms segregating in different chromosomal gene arrangements (shared polymorphisms). Certain sites are, nevertheless, associated statistically with particular gene arrangements. However, the resolution of restriction mapping techniques [using enzymes with either 6- or 4-nucleotide recognition sequences ("6-cutter" or "4-cutter" analysis)] does generally preclude assignment of a restriction site change to a single nucleotide.

Given that in the absence of recurrent mutation the presence of shared polymorphisms would be compatible either with a polyphyletic origin of inversions or with genetic recombination in inversion heterozygotes, it is important to unambiguously establish the existence of shared polymorphisms. Only complete information on a given region (as provided by direct sequencing) allows unambiguous assertions in this respect. For this, we have sequenced and compared the *rp49* region of *D. subobscura* from chromosomes differing in gene arrangement. The region including *rp49*, a single-copy gene which codes for ribosomal protein 49, has been chosen for the present study because in *D. subobscura* it is found within various inverted regions of the O chromosome. The data have also allowed us to discuss the relative age of different inversions.†

MATERIAL AND METHODS

Fly Samples. Ten lines of *D. subobscura* (one from Helsinki, Finland; eight from Barcelona, Spain; and one from Raíces, Canary Islands, Spain) were used (Table 1). Strains from Helsinki (H27) and Raíces (RA111), kindly provided by R. de Frutos (Universitat de València, Spain), had been made homozygous by sib mating. Strains from Barcelona are a subset of the 49 O chromosome lines that had been previously studied for the *rp49* region by 4-cutter analysis (9). For each chromosomal class, these lines were chosen at random (see ref. 9).

***In Situ* Hybridization.** Cytological location of the *rp49* region in the different heterokaryotypes was determined by *in situ* hybridization (11).

Sequencing. The *rp49* region of nine alleles (one allele from Raíces and eight from Barcelona) was amplified by the polymerase chain reaction (PCR) and sequenced; the 10th allele had been sequenced after its cloning (12). Genomic DNA was CsCl-purified (13). A 1.6-kb region, including the *rp49* gene as well as its 5′ and 3′ flanking regions, was amplified (14). Amplification primers were designed according to Rozas (15). Amplification primers as well as amplified DNA were treated according to Higuchi and Ochman (16) in order to obtain single-stranded DNA for each strand. Both strands were sequenced by the dideoxy method using modified T7 DNA polymerase. Eleven primers separated by about 250 nucleotides along the amplified region were used to determine the sequence. Sequences were aligned by using Staden's programs (17) and subsequently were manually aligned to minimize the number of nucleotide substitutions. Nucleotides are numbered according to Aguadé (12).

RESULTS

Chromosomal Polymorphism. Table 1 shows the chromosomal gene arrangement of each allele. Chromosomal gene arrangements have been grouped in different chromosomal

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*To whom reprint requests should be addressed.

†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X72646–X72654).

Table 1. Description of the strains used

Line	Chromosomal gene arrangement	Chromosomal class	Haplotype number
H27	O_{st}	$O_{[st]}$	
B1	O_{st}	$O_{[st]}$	16(1)
B2	O_{st}	$O_{[st]}$	10(6)
B3	O_{st}	$O_{[st]}$	5(1)
B4	O_{3+4+7}	$O_{[3+4]}$	7(1)
B5	O_{3+4}	$O_{[3+4]}$	43(16)
B6	O_{3+4}	$O_{[3+4]}$	8(2)
T7	O_{3+4}	$O_{[3+4]}$	
B8	O_{3+4+8}	$O_{[3+4+8]}$	39(1)
B9	O_{3+4+8}	$O_{[3+4+8]}$	2(3)

Haplotype number refers to restriction map haplotypes previously described (9). Line H27 is from Helsinki, line T7 is from Raíces, and the rest are from Barcelona.

classes according to the location of the *rp49* region (9, 18). Within any chromosomal class the *rp49* region has the same location, and consequently crossing over should not be prevented in homokaryotypes within chromosomal class. When the *rp49* region is considered, $O_{[st]}$ and $O_{[3+4]}$ classes differ by two inversions, $O_{[st]}$ and $O_{[3+4+8]}$ by three, and $O_{[3+4]}$ and $O_{[3+4+8]}$ by one. Fig. 1 shows the location of the *rp49* region in the three pairwise heterokaryotypes. Only in heterokaryotypes between $O_{[3+4]}$ and $O_{[3+4+8]}$ is the *rp49* region located rather centrally in the inversion loop; in the other heterokaryotypes it is located very close to one of the inversion breakpoints.

Nucleotide Substitutions. Fig. 2 summarizes the nucleotide sequence variation observed in the *rp49* region for the 10 alleles studied from *D. subobscura*. Out of 1475 nucleotides compared, 56 were polymorphic, and all but one (position 524) were segregating for only 2 nucleotides. Twenty of these 56 polymorphisms were present more than once in the sample.

Table 2 gives the distribution of nucleotide polymorphism in different functional regions. The three nucleotide polymorphisms detected in the exons are silent or synonymous. When all sites are considered, significant heterogeneity in the distribution of polymorphisms can be detected among the 5′ flanking, coding, and 3′ flanking regions ($\chi^2 = 15.01$ with df = 2, $P = 0.0005$); the coding region is less variable. When only silent sites are considered, no heterogeneity can be detected ($\chi^2 = 0.65$ with df = 2, $P = 0.721$), an indication of selective constraint against amino acid substitutions.

Table 3 shows the distribution of polymorphisms in the different chromosomal classes both for the 8 alleles from Barcelona and for all 10 alleles. The number of polymorphisms is highest in $O_{[3+4]}$ and lowest in $O_{[st]}$, with $O_{[3+4+8]}$ being intermediate, both when all polymorphisms and when only nonunique polymorphisms are considered. Estimated polymorphism and nucleotide diversity (19) are lowest for $O_{[st]}$ (Table 4).

Fig. 3 shows a network of the 10 alleles sequenced for the *rp49* region. Alleles within a chromosomal class have been connected according to the number of differences. Loops are an indication of recombination under the assumption of no back or parallel mutation; they represent the existence of the four gametic types for a pair of polymorphic sites located on contiguous sides of a loop. Within chromosomal class $O_{[st]}$, for example, a recombination event is inferred between positions 251 and 464. Recombination among chromosomal classes is indicated in Fig. 3 only by the presence of shared polymorphisms—i.e., polymorphisms segregating in different chromosomal classes. The total number of shared polymorphisms both for the alleles from Barcelona and for the overall 10 alleles is presented in Table 3.

Length Variation. In the present study seven length polymorphisms have been detected; four of these are unique. All polymorphisms are located in noncoding regions. Some length polymorphisms are located in regions with tandemly repeated short motifs. In the region between nucleotides 83 and 110 consisting of TTG repeats, four length variants have been detected, all of them multiples of 3 bp. A study of this region by 4-cutter analysis (9) had detected seven different variants in this same region differing also in multiples of 3 bp. In the 62-bp intron, two of the four length polymorphisms detected represent length variants differing in the number of either AATGG or AATGT repeats. Because slipped-strand mispairing might be the mechanism implicated in the generation of these polymorphisms (20), these polymorphisms were not considered in building the network connecting the sequenced alleles.

DISCUSSION

Unique vs. Multiple Origin of the Chromosomal Inversions. It is generally accepted that a particular chromosomal inversion has a unique origin (see ref. 21). However, a multiple origin of inversions has been sometimes considered, given the nonrandom distribution of inversion breakpoints along the chromosome (22, 23) and given that transposable elements can induce chromosome rearrangements (24, 25). Nevertheless, the data are consistent with a monophyletic origin and there is no compelling evidence for multiple origins.

Our results for the *rp49* region can also be interpreted by assuming a unique origin of inversions. First, some polymorphisms segregate differentially in different chromosomal classes. Moreover, in a phylogenetic tree built by parsimony (26), all *rp49* sequences within the $O_{[st]}$ chromosomal class appear to be clustered (data not shown); although this does not happen for chromosomal class $O_{[3+4+8]}$ (see below). Second, within a particular chromosomal class, no geo-

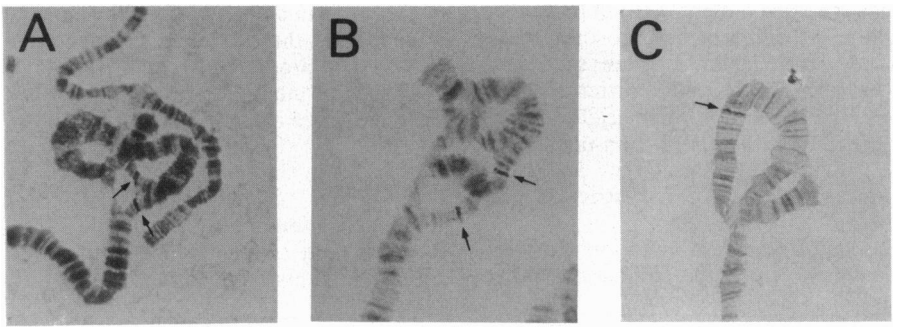

FIG. 1. Cytological location of the *rp49* region (indicated by arrows) in the different heterokaryotypes. (*A*) O_{st}/O_{3+4}. (*B*) O_{st}/O_{3+4+8}. (*C*) O_{3+4}/O_{3+4+8}.

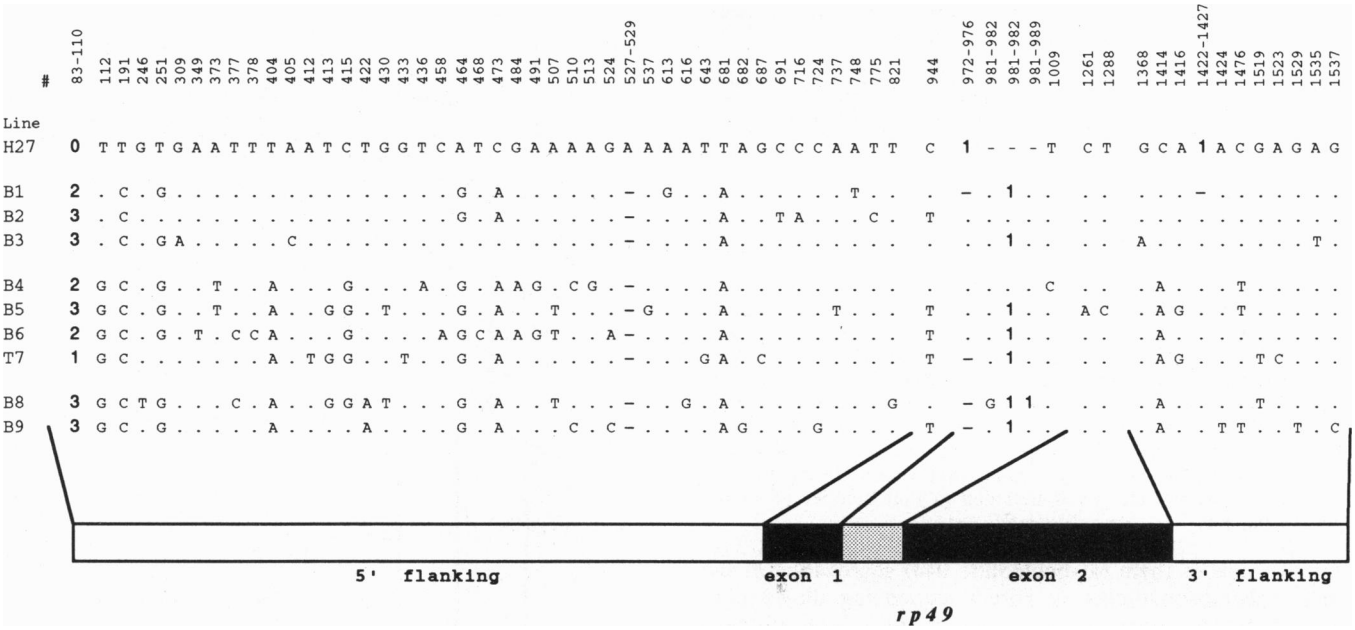

FIG. 2. Nucleotide polymorphisms at the *rp49* region of *D. subobscura*. Polymorphisms are numbered according to the sequence of line H27 (12). Dots indicate nucleotides identical to the H27 sequence. Dashes indicate deletions. Insertions or deletions longer than 1 bp are indicated by numbers. Polymorphism between nucleotides 83 and 110: 0, CTG; 1, deletion of CTG and insertion of TTGTTG; 2, deletion of CTGTTGTTG; 3, deletion of CTG. Polymorphism between nucleotides 972 and 976: 1, AATGG. Polymorphism between nucleotides 981 and 982: 1, insertion of AATGT. Polymorphism between nucleotides 981 and 989: 1, substitution of TGGTATGCT for AAAAACC. Polymorphism between nucleotides 1422 and 1427: 1, TTA.

graphic heterogeneity in the distribution of haplotypes and restriction-site polymorphisms was detected (9, 27).

Heterozygosity in Different Chromosomal Gene Arrangements. Nucleotide heterozygosity in inverted and noninverted chromosomes could provide valuable information about the history of chromosomal inversions. If variation in a given region of an inversion were neutral, genetic recombination (by double crossover or gene conversion) would tend to homogenize the genetic content of different gene arrangements, whereas mutation would tend to mediate its differentiation. Although crossing over seems to be highly suppressed in inversion heterozygotes, especially in regions close to the breakpoints, levels of gene conversion do not seem to be affected in structural heterozygotes (6).

If the rate of gene conversion were lower than the neutral mutation rate (case *a*), mutation would drive the process and, at equilibrium, heterozygosity would be approximately equal to $4N_e\mu$, where N_e is the effective population size and μ is the neutral mutation rate (28). Therefore, if the different gene arrangements had attained equilibrium, differences in heterozygosity would be an indication of different population sizes. However, the time required to attain equilibrium may be considerable (29). If gene arrangements were not yet in equilibrium, that gene arrangement with higher heterozygosity would be older.

If the rate of gene conversion were higher than the mutation rate (case *b*), heterozygosity in the new inversion would increase mainly by genetic exchange with other gene arrangements. At equilibrium, no differences in heterozygosity between different gene arrangements would be expected. On its way to equilibrium, heterozygosity would be higher in the older gene arrangement.

For the *rp49* region, case *b* would apply, as gene conversion seems to be playing an important role (see below). Differences in the estimates of heterozygosity within $O_{[st]}$ and within $O_{[3+4]}$, both by 4-cutter analysis and by direct sequencing, would therefore indicate that $O_{[3+4]}$ is older than $O_{[st]}$. This is in agreement with some previous reports (see ref. 9).

Transfer of Genetic Information Between Chromosomal Gene Arrangements. If inversions have a monophyletic origin and if transfer of genetic information between inverted segments during meiosis were suppressed, a particular gene would be expected to evolve independently in inverted and noninverted chromosomes. The effect of double crossover and gene conversion, which would slow the expected differentiation, has classically been considered negligible, especially in regions located very close to inversion breakpoints (as in the *rp49* region between $O_{[st]}$ and $O_{[3+4]}$). However, in our previous 4-cutter analysis of 107 lines at the *rp49* region (9), we detected several polymorphisms segregating in different chromosomal gene arrangements (or shared polymorphisms). Here, the sequence analysis of some of these lines has also revealed several shared polymorphisms (Table 3 and

Table 2. Distribution of nucleotide polymorphism in different functional regions

	5′	Exon 1	Intron	Exon 2	3′	Total
No. of nucleotide sites	798	93	62	309	213	1475
(no. silent)		(21)		(68)		
No. of polymorphic sites	42	1	1	2	10	56
Polymorphism frequency	0.0526	0.0108	0.0161	0.0065	0.0469	0.0380
(silent)		(0.0476)		(0.0294)		
Nucleotide diversity	0.0148	0.0060	0.0032	0.0013	0.0137	0.0108
(silent)		(0.0265)		(0.0059)		

Nucleotide diversity (π) was estimated according to Nei (19).

Table 3. Number of polymorphisms within chromosomal classes and number of shared polymorphisms between chromosomal classes

	$O_{[st]}$	$O_{[3+4]}$	$O_{[3+4+8]}$
$O_{[st]}$	15 (4)	2	1
	13 (4)	1	1
$O_{[3+4]}$		30 (13)	8
		23 (11)	7
$O_{[3+4+8]}$			18 (9)
			18 (9)

Upper values indicate the number of polymorphisms scored when all 10 sequences were considered, and lower values those when only the 8 alleles from Barcelona were considered. Data in the main diagonal indicate the total number of polymorphisms within each chromosomal class and also (in parentheses) the number of polymorphisms present only more than once in the whole sample. Polymorphisms shared by any two chromosomal classes are indicated above the diagonal. Polymorphism at position 944 shared by all three chromosomal classes is included in that number of shared polymorphisms.

Fig. 3), one of them (at nucleotide 944) segregating in the three chromosomal classes. Direct sequencing allows us to unambiguously assert the existence of equivalent polymorphisms segregating in different chromosomal classes.

In considering chromosomal classes $O_{[st]}$ and $O_{[3+4]}$, which differ by two overlapping inversions, each one including the *rp49* region, and where the hypothetical O_3 intermediate is not present at extant populations, our data would require two events of very low probability. If the existence of shared polymorphisms between $O_{[3+4]}$ and $O_{[st]}$ were due to polyphyletic origin, it would require that both the multiple inversion event connecting O_3 and O_{3+4} and the multiple event connecting O_3 and O_{st} had captured both variants of a particular polymorphism: the probability of these two events happening seems very low. Our data for $O_{[3+4]}$ and $O_{[st]}$, even if they were polyphyletic in origin, would therefore be most probably the effect of (*i*) parallel or back mutations or (*ii*) transfer of genetic information between inverted chromosomal segments by double crossover or gene conversion. For length polymorphisms located in regions with simple repetitive motifs, the effect of parallel or back mutation might be important; some length variants might have arisen independently in different chromosomal gene arrangements by slipped-strand mispairing (20). However, for nucleotide substitutions (in the absence of mutation hotspots) the effect of parallel or back mutation should be negligible. Therefore, our data provide evidence for transfer of genetic information between inversions. Additional results (unpublished data) showing some blocks of sites transferred from one inversion to another strongly support crossovers or gene conversion tracts. The transfer of genetic information between inversions would imply that the gene pool harbored by a particular chromosomal inversion is less well isolated from noninverted chromosomes than previously thought. Both gene conversion and double crossover can be responsible for genetic transfer; however, experiments (6) demonstrating that gene conversion occurs in inversion heterozygotes at frequencies comparable to those in homozygotes would favor the former mechanism.

Table 4. Estimates of the frequency of polymorphism and nucleotide diversity

	Frequency of polymorphism		Nucleotide diversity	
Class	Barcelona	Total	Barcelona	Total
$O_{[st]}$	0.0088	0.0102	0.0059	0.0054
$O_{[3+4]}$	0.0156	0.0203	0.0104	0.0110
$O_{[3+4+8]}$	0.0122	0.0122	0.0122	0.0122

Nucleotide diversity (π) was estimated according to Nei (19).

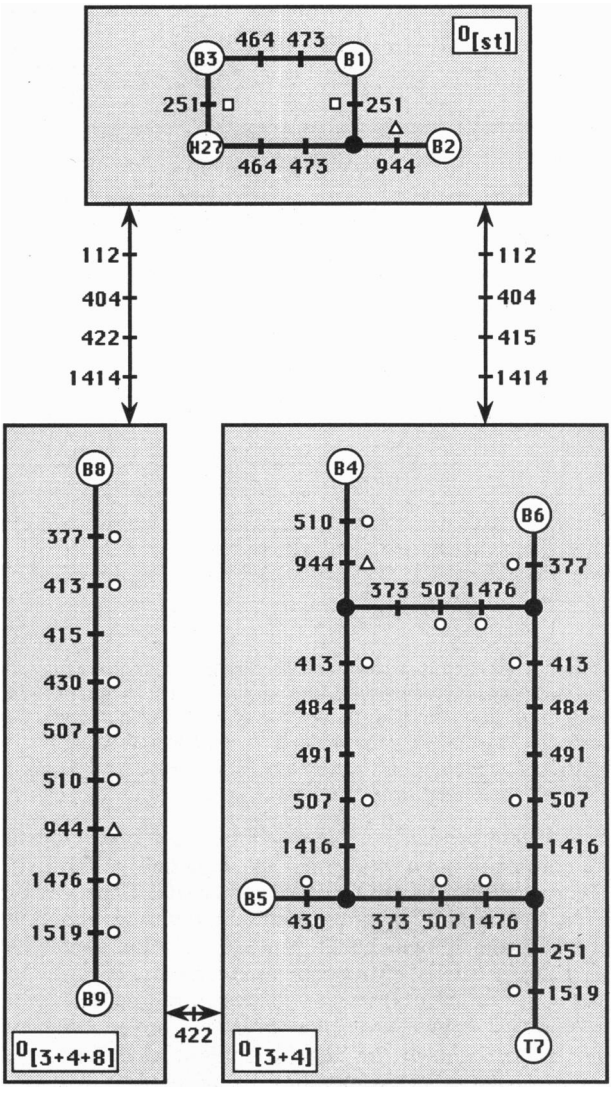

FIG. 3. Network of the 10 alleles sequenced for the *rp49* region. Numbers in circles indicate the different alleles sequenced. Alleles within the same chromosomal class have been grouped in big shaded boxes (see Table 1). Differences between alleles are indicated by their location in the sequenced region (unique polymorphisms have not been considered). The location of nucleotide differences between any two chromosomal classes is indicated in the connecting arrows. Polymorphisms shared by different chromosomal classes are indicated by the following symbols: □, between $O_{[st]}$ and $O_{[3+4]}$; ○, between $O_{[3+4]}$ and $O_{[3+4+8]}$; △, among $O_{[st]}$, $O_{[3+4]}$, and $O_{[3+4+8]}$.

Gene transfer seems higher between $O_{[3+4]}$ and $O_{[3+4+8]}$ than between $O_{[st]}$ and $O_{[3+4]}$; in fact, the $O_{[3+4+8]}$ sequences do not form a separate cluster from those within $O_{[3+4]}$ in the phylogenetic tree (data not shown). The location of the *rp49* region in the inversion loop varies in structural heterozygotes between $O_{[3+4]}$ and $O_{[st]}$ relative to $O_{[3+4]}$ and $O_{[3+4+8]}$ (Fig. 1); *rp49* is very close to one of the breakpoints in the former but in the middle of the inversion loop in the latter. If $O_{[3+4+8]}$ proved not to be very old and these preliminary results on higher gene conversion between $O_{[3+4]}$ and $O_{[3+4+8]}$ were confirmed, they might indicate that gene conversion in structural heterozygotes might not be independent of location (i.e., lower for genes located close to breakpoints). Alternatively, the assumption of a monophyletic origin of inversions might not be general, and the present results point to a polyphyletic origin of inversion 8.

There are only a few reports relating chromosomal and DNA variation in natural populations, and except for the present study at the *rp49* region, the rest have studied DNA variation by restriction map analysis (8–10, 30). Both Aquadro *et al.* (30), and Aguadé (8) have reported the existence of several polymorphisms in the *Adh* region of *D. melanogaster* segregating in both standard and inverted—*In(2L)t*—chromosomes. The *Adh* gene is located outside the inversion but very close to one of the breakpoints. In the *Amy* region of *D. pseudoobscura* (10) there are a few shared polymorphisms but the chromosomal gene arrangements are well differentiated. In fact, phylogenies based on DNA variation at this region and on chromosomal inversions are congruent unlike the other regions studied. Several neutral factors might account for the difference between the *rp49* region in *D. subobscura* and the *Amy* region in *D. pseudoobscura*. First, the region including the *Amy* gene in *D. pseudoobscura* may have a lower gene conversion rate than that including the *rp49* gene in *D. subobscura*. Second, the chromosomal polymorphism in the latter species might be older than that in *D. pseudoobscura*. Alternatively, some selective arguments might account for the observed difference. In fact, natural populations segregate for different *Amy* allozymes, while for the *rp49* gene there is no variation at the protein level. Selection might be operating at the protein level and contribute to the divergence of the *Amy* region between inversions. Sequencing data need to be gathered for allozyme genes like *Pep-1* and *Lap* in *D. subobscura*, located in the same chromosomal segment with *rp49*. If these genes showed a different pattern of differentiation between gene arrangements than the *rp49* region, this would point to natural selection as an important force acting on allozyme variation despite the existence of gene flow between inversions.

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