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Molecular organization of the *Drosophila melanogaster Adh* chromosomal region in *D. repleta* and *D. buzzatii*, two distantly related species of the *Drosophila* subgenus

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Abstract

The molecular organization of a 1.944-Mb chromosomal region of *Drosophila melanogaster* around the *Adh* locus has been analyzed in two *repleta* group species: *D. repleta* and *D. buzzatii*. The extensive genetic and molecular information about this region in *D. melanogaster* makes it a prime choice for comparative studies of genomic organization among distantly related species. A set of 26 P1 phages from *D. melanogaster* were successfully hybridized using fluorescence *in-situ* hybridization (FISH) to the salivary gland chromosomes of both *repleta* group species. The results show that the *Adh* region is distributed in *D. repleta* and *D. buzzatii* over six distant sites of chromosome 3, homologous to chromosomal arm 2L of *D. melanogaster* (Muller's element B). This observation implies a density of 2.57 fixed breakpoints per Mb in the *Adh* region and suggests a considerable reorganization of this chromosomal element via the fixation of paracentric inversions. Nevertheless, breakpoint density in the *Adh* region is three times lower than that estimated for *D. repleta* chromosome 2, homologous to *D. melanogaster* 3R (Muller's element E). Differences in the rate of evolution among chromosomal elements are seemingly persistent in the *Drosophila* genus over long phylogenetic distances.

Introduction

The comparison of the genomic organization of small chromosomal regions among distantly related species within the genus *Drosophila* provides valuable information for understanding the evolutionary forces acting on the localization, order and distance of genes along the chromosomes (Hartl & Lozovskaya 1994). The evolutionary conservation of genes clusters is often taken as evidence for functional constraint, implying that natural selection has not permitted changes in the organization of the cluster to be fixed (Martinez-Cruzado *et al.* 1988, Hooper *et al.* 1992,

Maier *et al.* 1993, Stathakis *et al.* 1995). However, this hypothesis is usually not subjected to any rigorous statistical test because rates of chromosomal evolution are usually not available. Some chromosomal regions that have been studied, e.g. the HOX gene complexes (Randazzo *et al.* 1993, Von Allmen *et al.* 1996), may not be representative of the genome as a whole. Very few comparative mapping studies have focused on the organization of more 'typical' chromosomal regions (Nurminsky *et al.* 1996, Vieira *et al.* 1997, Ranz *et al.* 1999). In these cases, the results show an extensive reshuffling of gene content and are usually compatible with the hypothesis that the gene organization of chromosomes is the result of random fixation of chromosomal rearrangements. Clearly, more studies are needed to draw a balanced view of the role that chance and natural selection play on the evolution of the organization of chromosomes.

The chromosomal region around the *Adh* locus of *D. melanogaster* has been genetically characterized to a greater degree than any other comparable region in any metazoan. Genetic analysis began in the early 1960s with the recovery of an Adh^{-} deletion (Grell *et al.* 1968). Since then 73 genes and 850 different mutant alleles, as well as 86 inversions, 109 translocations, 317 deletions and 40 duplications, have been defined in this region. This detailed genetic analysis was the reason to choose this region for the first experiment in megabase sequencing and sequence analysis in *Drosophila* (Ashburner *et al.* 1999). The entire region extends from 34C4 to 36A2 on chromosome arm 2L, is 2.9 Mb long and has been sequenced from a series of overlapping P1 and BAC clones. Analysis of the sequence now reveals 233 genes (222 protein coding genes and 11 tRNAs), some by direct experiment but the majority as ORFs predicted by a variety of algorithmic methods. The average gene density is one protein coding gene per 13 kb but there is an enormous variation in density along the region. A significant proportion ($\sim 17\%$) of the total number of protein coding genes identified in this region are arranged in clusters, suggesting extensive tandem duplication. Seventeen cases of nested genes have been identified. The density of transposable elements is one element every 171 kb.

The extensive genetic and molecular information about the *D. melanogaster Adh* region makes it a prime choice for comparative studies of the genomic organization among distantly related species. We have chosen *D. repleta* and *D. buzzatii* for this comparison for three reasons. (1) They belong to the large *repleta* species group in the subgenus *Drosophila* (Wasserman 1992) which diverged 40–62 million years ago from *D. melanogaster*, which is in the subgenus *Sophophora* (Beverly & Wilson 1984, Russo *et al.* 1995). (2) The molecular evolution of the *Adh* genes has been already studied in several *repleta* group species, including *D. buzzatii* (Sullivan *et al.* 1990, Menotti-Raymond *et al.* 1991, Schafer 1992, Sullivan *et al.* 1994, Russo *et al.* 1995, Begun 1997, Betrán & Ashburner, in preparation). (3) An ongoing project to compare the evolution rates of the different chromosomes is being carried out in our laboratory (Barcelona) using the two *repleta* group species (Ranz *et al.* 1997, Ruiz *et al.* 1997, Ranz *et al.* 1999, Ranz JM, Casals F, Ruiz A, in preparation).

We have analyzed the *D. melanogaster* region extending from 34C4 to 35D4 in chromosomal arm 2L, a region 1.944 Mb long that includes the *Adh* locus. A set of 26 P1 phages were successfully hybridized using fluorescence *in-situ* hybridization (FISH) to the salivary gland chromosomes of the two *repleta* group species. The 26 P1 clones were selected among the 38 sequenced P1 phages comprising the *Adh* contig in such a way as to provide a full coverage of the region. The results show that the *Adh* region is distributed in *D. repleta* and *D. buzzatii* over six distant chromosomal sites of chromosome 3, homologous to chromosomal arm 2L of *D. melanogaster*. This observation implies a considerable reorganization of this chromosomal element via the fixation of paracentric inversions. However, the estimated breakpoint density in the *Adh* region is three times lower than that estimated for *D. repleta* chromosome 2 (homologous to *D. melanogaster* 3R). Therefore, differences in the rate of evolution among chromosomal elements are seemingly persistent in the *Drosophila* genus over long phylogenetic distances.

Materials and methods

Stocks

The following species and stocks were used: one stock of *D. melanogaster* (Canton S) homokaryotypic for the standard arrangements in all chromosomes (Lemeunier & Aulard 1992); one stock of *D. repleta* (1611.6) chromosomally monomorphic (Wasserman 1992) from the National *Drosophila* Species Resource Center, Bowling Green; and one stock of *D. buzzatii* (39.13st) homokaryotypic for the *2st* chromosomal arrangement (Ruiz & Wasserman 1993) and isolated by repeated sib-mating from wild flies collected in Carboneras, Spain (Betrán *et al.* 1998).

Probes

Twenty-six clones from the *D. melanogaster* Berkeley Drosophila Genome Project's P1 library were used as probes. All the clones came from the *D. melanogaster* 34C4–35D4 region (Table 1 & Figure 1). The average insert size (±SD) of these clones, excluding DS09194, is 53.5 kb (±26.8). Clone DS09194 was sequenced together with two other clones and thus its sequence is not available separately (BDGP 1999). Several subclones from clone DS00941 were also used as probes (see below). The average insert size of these subclones is 3.5 kb (BDGP 1999).

Table 1. List of the 26 P1 phages from the *Adh* contig used in this study (BDGP 1999)

Clone	Known genes	Sequence length (bp)	Chromosomal localization		Length of conserved segments (kb)
			D. melanogaster	*D. repleta/D. buzzatii*	
DS01368	None	58 484	34C	E4c-d	≥143
DS08284	p38b	39 042	34D	E4c-d/G3h	
DS00941	Sos, black, tamas, Sop2, Orc5, MtPo1B, RpII33	81 023	34D	G3h/E3c-d	50
DS01514	None	26 925	34F	E3c	
DS00131	None	5557	34F	E3a-c	
DS01759	None	49 500	34F	E2g-h	531
DS01523	None	59 991	34F	E2g-h	
DS01652	None	28 374	34F	E2g-h	
DS01068	wb, Rab14, spel1	48 158	35A	E2g-h	
DS06238	spel1, ppk	84 883	35B	G1b	
DS08340	None	25 043	35B	G1b	
DS01160	None	9508	35B	G1b	366
DS01486	osp, Adh, Adhr	81 562	35B	G1ab	
DS07721	None	79 993	35B	G1a	
DS00810	None	23 637	35B	E2c-d	
DS06874	None	85 139	35B	E2cd	
DS03431	Mst35Ba, Mst35Bb	51 989	35B	E2a-b	
DS01219	None	63 495	35B	E2a-b	520
DS00929	Su(H), BcDNA:LD22017, TfIIS	79 884	35C	E2ab	
DS04929	vas, stc	47 171	35C	E1f-g	
DS09194	None	139 666*	35C	E1f-g	
DS05639	None	7840	35D	C4b	
DS07851	esg	87 747	35D	C4b	
DS03023	None	84 043	35D	C4b	≥331
DS01845	sna	73 739	35D	C4b	
DS04862	None	53 670	35D	C4b	

* Combined length of P1 clones: DS03192, DS09194 and DS07295.

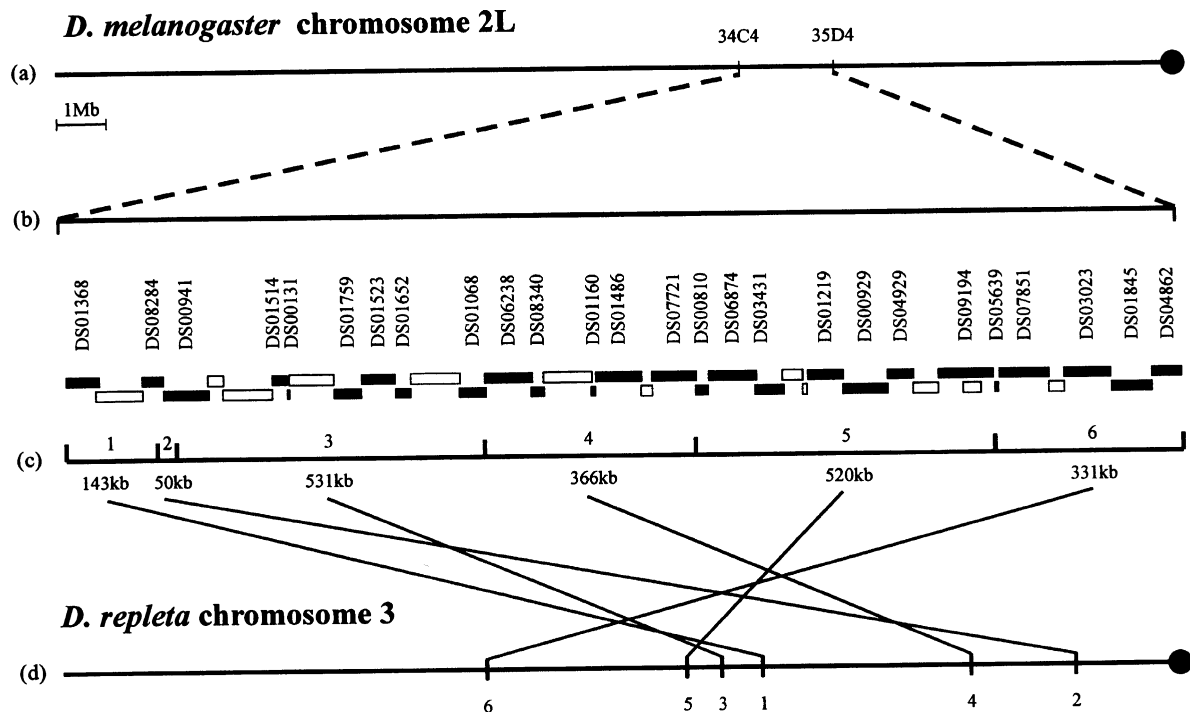

Figure 1. (**a**) Relative position of the 34C4–35D4 region in the 2L chromosome of *D. melanogaster*. (**b**) Enlarged representation of this region with the P1 clones comprising the *Adh* contig shown as rectangles. Solid rectangles represent P1 clones hybridized in *D. repleta* and *D. buzzatii*. Open rectangles represent P1 clones not hybridized in this study. (**c**) Conserved segments and their lengths. (**d**) Schematic map of chromosome 3 of *D. repleta* showing the hybridization sites of the P1s.

Fluorescence in-situ *hybridization*

Polytene chromosomes squashes, prehybridization and hybridization were carried out as described by Montgomery *et al.* (1987). DNA from subclones 8_g3, 2_f12 and 4_e12 were mixed and labeled as a single labeling reaction. The same was done with subclones 5_a4, 6_a4 and 4_h6 and with subclones 7_e10, 1_a9, 4_e10, 3_a5 and 4_f7. Probes were labeled by nick translation with either biotin-labeled or digoxigenin-labeled dUTP. Hybridization of biotinylated probes was detected with FITC-conjugated streptavidin. Digoxigenin-labeled probes were immunolocalized by means of fluorescently labeled (Texas red or Cy3) anti-digoxigenin antibodies. Slides were examined using a Bio-Rad MRC 600 laser-scanning confocal microscope. Images were collected using a Zeiss Axioplan fluorescence microscope equipped with a 100 W mercury source, merged using CoMOS software (Bio-Rad) and then opened in Photoshop (Adobe). This differential labeling and detection allowed the simultaneous hybridization of two different P1 clones (or two different subsets of subclones) with the same polytene chromosome squash (see Figure 2).

Chromosome maps

Hybridization signals were localized on the polytene chromosomes using the following cytological or photographic maps: *D. melanogaster* (Lefevre 1976), *D. repleta* (Wharton 1942) and *D. buzzatii* (Ruiz & Wasserman 1993). The band notation for *D. repleta* and *D. buzzatii* is the same because the cytological map of the latter species is a cut-and-paste reconstruction of the *D. repleta* map.

Data analysis

Let L_R be the length of the analyzed chromosomal region and L_T that of the entire chromosomal element (both measured in Mb in the reference

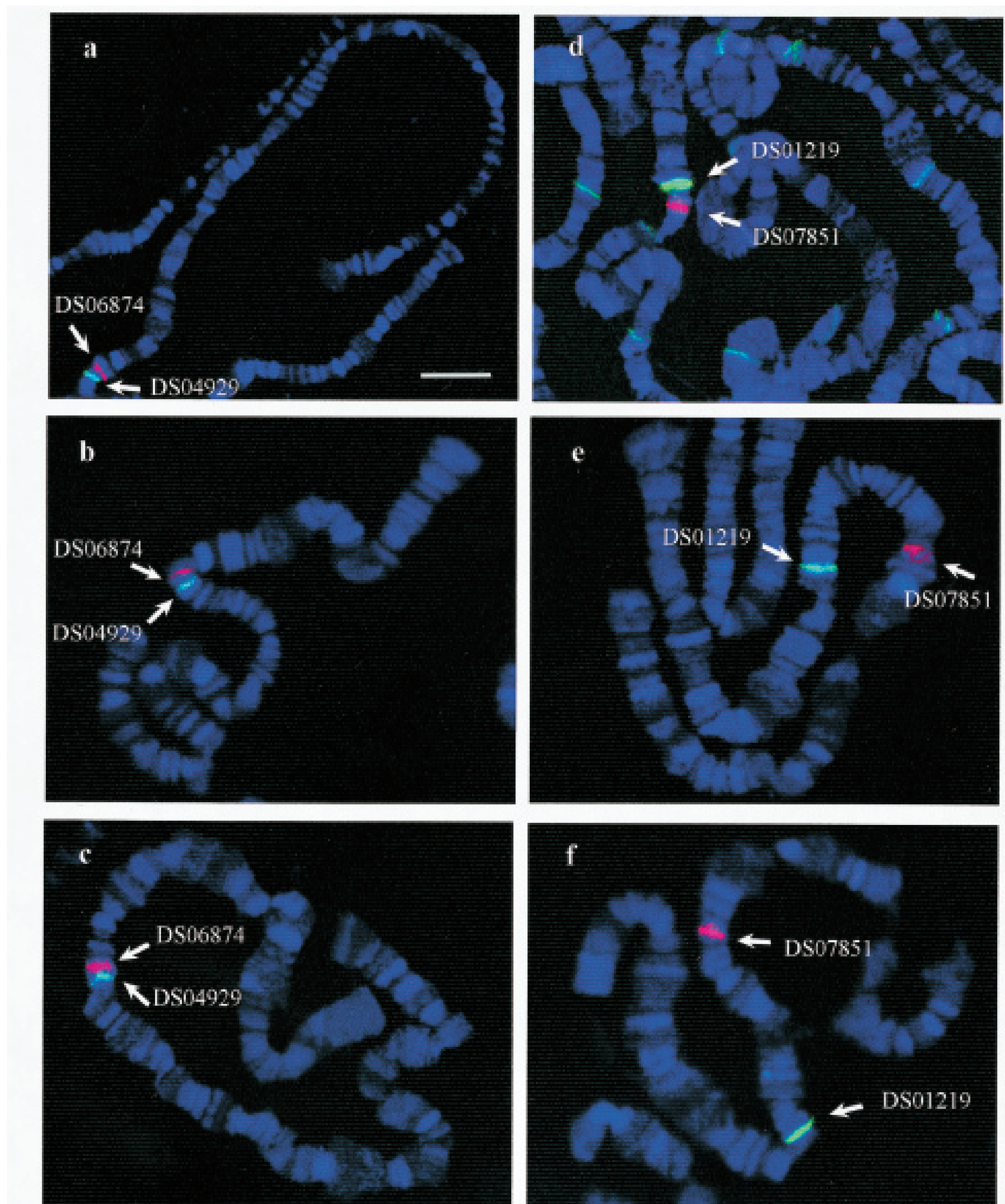

Figure 2. Hybridization of the P1s DS06874, DS04929, DS07851 and DS01219 in *D. melanogaster* (**a** and **d**), *D. repleta* (**b** and **e**) and *D. buzzatii* (**c** and **f**). Note that DS06874 and DS04929 map close to each other in *D. melanogaster* (**a**) and also in *D. repleta* (**b**) and *D. buzzatii* (**c**) because both belong to the same conserved segment. DS07851 and DS01219 map close to each other in *D. melanogaster* (**d**) but well separated in *D. repleta* (**e**) and *D. buzzatii* (**f**) because they pertain to different conserved segments. The weak secondary signals produced by DS01219 in *D. melanogaster* (**d**) may be due to repetitive sequences such as the transposable element yoyo, one copy of which is present in this clone.

species, *D. melanogaster*). If fixed breakpoints are distributed at random, the probability that a breakpoint falls within the chromosomal region is equal to its relative length, $l = L_R/L_T$, and the probability of observing N_R breakpoints within the region out of N breakpoints fixed in the entire chromosomal element follows the binomial distribution: $[l + (1 - l)]^N$ with mean Nl and variance $Nl(1 - l)$. Therefore, N can be estimated as N_R/l and the density of fixed inversion breakpoints in the analyzed chromosomal region as $d = N_R/L_R$. In addition, the variances of these estimates can be calculated as follows:

$$\mathrm{Var}(N_R) = N_R(1 - l)$$
$$\mathrm{Var}(N) = (1/l)^2 N_R(1 - l)$$
$$\mathrm{Var}(d) = (1/L_R)^2 N_R(1 - l)$$

Confidence limits for N_R, N and d can be derived from the tabulated unbiased confidence limits for proportions (Sokal & Rohlf 1995, p. 156).

Results

The *D. melanogaster* chromosomal region 34C4–35D4 is entirely contained in 38 overlapping P1 phages, which constitute the *Adh* contig (Figure 1). In order to fully characterize the organization of this region in *D. repleta* and *D. buzzatii* with the minimum experimental effort, we used the following strategy. Firstly, every third P1 clone in the ordered contig was *in-situ* hybridized to the chromosomes of both *repleta* group species. When two consecutive clones mapped to a different chromosomal site, then the two intervening P1 clones were also hybridized in order to get a more accurate localization of the implied breakpoint. On the other hand, when two consecutive clones mapped to the same chromosomal site, we considered that they belong to a conserved chromosomal fragment comprising also the non-hybridized intervening P1 clones (any alternative explanation would be highly unlikely). Finally, when two clones show a considerable sequence overlap (determined by BLAST analysis), only one of them was hybridized. A full coverage of the region was achieved in this way by the successful hybridization of 26 P1 clones (Figure 1). We are quite confident that all conserved chromosomal fragments and fixed inversion breakpoints in the region have been detected.

All the clones hybridized to chromosome 3 of *D. repleta* and *D. buzzatii*, which is homologous to chromosomal arm 2L of *D. melanogaster* (Muller's element B) (Wasserman 1992, Ranz *et al.* 1997). In addition all hybridization sites were identical in the two species (Table 1 & Figure 2). Chromosome 3 of *D. buzzatii* is homosequential with that of *D. repleta* except for a small inversion, *3b*, which is located in the middle region of the chromosome (Wasserman 1992, Ruiz & Wasserman 1993). None of the 26 P1 phages hybridized inside or close to the *3b* inversion. All P1 clones but two gave a single hybridization signal (see Figure 2 for a sample of representative hybridization signals) which points to the localization of the homologous regions in *D. repleta* and *D. buzzatii*. Two adjacent clones, DS08284 and DS00941, produced two signals each (Figure 1 and Table 1). Both clones share one of the hybridization sites, G3h. DS08284 shows one additional signal in E4c-d, a site which is coincident with that of the preceding clone (DS01368). Likewise, DS00941 produced a further signal in E3c-d, which is close to the hybridization site of the next six P1 clones (see Table 1). Thus, given the clone sequence in *D. melanogaster* (Figure 1) and the localization of the hybridization signals in the two *repleta* group species (Table 1) the most likely explanation for the two double signals is the presence of a fixed breakpoint in each clone. To further corroborate this hypothesis, we hybridized several subclones from both ends of P1 clone DS00941. The insert of this P1 has been subcloned into 28 plasmids (BDGP 1999). First of all we hybridized the three subclones from the 5′ end and the three subclones from the 3′ end of this P1 to the polytene chromosomes of *D. repleta*. The subclones from the 5′ end of the P1 (8_g3, 2_f12 and 4_e12) gave a single hybridization signal in polytene band G3h. The subclones from the 3′ end (5_a4, 6_a4 and 4_h6) gave multiple signals. Then we hybridized the 5 subclones adjacent to these three subclones (7_e10, 1_a9, 4_e10, 3_a5 and 4_f7) and they gave a single hybridization signal in the polytene

Table 2. Estimated number of inversion breakpoints fixed between *D. melanogaster* and *D. repleta* (or *D. buzzatii*) in Muller's elements B and E. Data from this work and Ranz JM, Casals F, Ruiz A (in preparation).

Muller's element	B	E	E
Chromosomal region	34C4-35D4	83E1-84E1 86A4-E2 95A1-96A23 97B1–E6	80–100
Size (Mb)	1944	4.938	28
Number of markers	26	100	161
Number of breakpoints	5	31	238
Density (breakpoints/Mb)	2.57	6.28	8.50
SD	1.10	1.02	1.08
95% Confidence limits	1.03–5.57	4.48–8.54	6.35–10.65

band E3c-d. The physical separation of the two signals provides a firm basis for our interpretation.

The 1.944-Mb region around the *Adh* locus in *D. melanogaster* is distributed over 6 distant sites of chromosome 3 in *D. repleta* and *D. buzzatii* (Figure 1). P1 clone DS01486 which contains the genes *outspread* (*osp*), *Alcohol dehydrogenase* (*Adh*) and *Adh-related* (*Adhr*) mapped to band G1ab, in good agreement with the previous localization of *Adh* (Labrador *et al.* 1990) and *osp* and *Adhr* (Betrán & Ashburner, in preparation). The presence of sequences homologous to the *Adh* region in six different sites implies that 5 rearrangement breakpoints have been fixed in this region during the divergence of *D. melanogaster* and *D. repleta* (or *D. buzzatti*). Therefore, the estimated breakpoint density for this region is 2.57 breakpoints per Mb (Table 2) and the average size of the conserved chromosomal fragment is 324kb.

Discussion

By mapping DNA clones from a particular chromosomal region in distantly related species, valuable information on the evolution of its molecular organization can be obtained. In the genus *Drosophila*, such studies have previously focused on the evolution of gene clusters and gene complexes (e.g. Hooper *et al.* 1992). In contrast, little work has been done on the evolution of more 'typical' chromosomal regions, and what has been done has usually been limited to a number of markers scattered along the region studied (Nurminsky *et al.* 1996, Vieira *et al.* 1997, Ranz *et al.* 1999). Here, we have analyzed the evolution of the *D. melanogaster Adh* region in *D. repleta* and *D. buzzatti*, two distantly related species belonging to the *repleta* species group (subgenus *Drosophila*). We have taken advantage of the fact that this region was the first to be completely sequenced and annotated in *D. melanogaster*. We have hybridized 26 P1 clones, covering the entire region, to the salivary gland chromosomes of the *repleta* group species. The results allow us to: (1) determine the number of inversion breakpoints fixed between *D. melanogaster* and the two *repleta* group species and estimate the rate of chromosomal evolution; (2) locate fairly accurately the inversion breakpoints along the DNA sequence; and (3) estimate the size of the conserved chromosomal fragments and test the null hypothesis that they have been produced as a result of random fixation of inversions.

All P1 clones used in this study come from the *Adh* region of *D. melanogaster* chromosome arm 2L (Muller's element B) and hybridized to the homologous chromosome 3 of the *repleta* group species. This result corroborates once more (Sturtevant & Novitski 1941, Steineman 1982, Steineman *et al.* 1984, Loukas & Kafatos 1986,

among others) the preservation of the gene content of the major chromosomal elements within the genus *Drosophila* (Muller 1940). However, when the order and spacing of the different mapped clones between *D. melanogaster* and the *repleta* species is compared (Figure 1), we see that it has changed remarkably. The most likely explanation for this result is the fixation in this chromosome of several paracentric inversions during the divergence of these species (Segarra & Aguadé 1992, Schafer *et al.* 1993, Kress 1993, Hartl & Lozovskaya 1994, Segarra *et al.* 1995, Ranz *et al.* 1997, 1999).

The *Adh* region of *D. melanogaster* is represented by 6 different chromosomal sites in the two *repleta* group species. This result implies that 5 inversion breakpoints have been fixed during the evolutionary divergence of these species, i.e. a density of 2.57 breakpoints per Mb (Table 2). This figure contrasts with the breakpoint density estimated for Muller's element E comparing the *D. repleta* chromosome 2 with the homologous chromosomal arm 3R of *D. melanogaster* (Ranz *et al.* 1999, Ranz JM, Casals F, Ruiz A, in preparation). In this element, four different regions (about 1 Mb long each) have been now analyzed in detail. The four regions were homogeneous giving an average of 6.28 breakpoints per Mb (Table 2). This is a minimum estimate for the number of fixed inversion breakpoints because the mapped clones did not provide a complete coverage of these chromosomal regions. The total number of inversions fixed in Muller's element E has also been estimated using all the available information for *D. repleta* chromosome 2 (161 DNA markers) by means of a maximum likelihood method (Ranz *et al.* 1997). The resulting value, 119 inversions, implies an average density of 8.50 breakpoints per Mb for this element, which is 28 Mb long in *D. melanogaster* (Table 2). Therefore, our results indicate a breakpoint density for the *Adh* region of element B three times lower than that for element E.

This difference in breakpoint density is most likely due to the different rates of evolution of the two Muller's elements and not to the particular chromosomal region analyzed here. If the *Adh* region, which is typical in many respects (Ashburner *et al.* 1999), were indeed representative of chromosomal arm 2L (which is 23 Mb long), ~30 inversions would have been fixed in element B (vs. 119 in element E). It has been previously noted that, within the *D. repleta* species group, chromosome 2 (element E), with approximately 23% of the euchromatin, harbors 76 of the 119 fixed inversions while chromosome 3 (element B), with 21% of the euchromatin, has only 18 fixed inversions (Wasserman 1992). Our results suggests that this pattern may also hold between phylogenetically very distant species which diverged 40–62 million years ago.

To localize the five inversion breakpoints in the sequence of the *Adh* region (see Figure 1 in Ashburner *et al.* 1999) and on the Ribbon map of this region available at ⟨http://www.fruitfly.org⟩, we took into account the sequence overlap and the gene content of the P1 clones as well as the hybridization signals that they produced (and the relative intensity in the case of DS08284 and DS00941 which give two signals each). We assume that breakpoints of fixed inversions are more likely to be located in the DNA between genes (Cirera *et al.* 1995, Cáceres *et al.* 1999) than within transcriptional units (as in Schneuwly *et al.* 1987). The two consecutive P1 clones, DS01068 and DS06238, gave signals in E2g-h and G1b, respectively. This places the breakpoint in the 859–872-kb interval between genes *spell* and *ppk*. Another inversion breakpoint is localized between the P1 clones, DS07721 and DS00810, which hybridized in G1a and E2c-d, respectively. This inversion breakpoint could be in the 1225–1229-kb interval between the genes *BG:DS07721.6* and *BG:DS00810.1*. The third inversion breakpoint is that localized between clones DS09194 and DS05639, which also hybridize at different chromosomal sites in *D. repleta* and *D. buzzatii*. We tentatively localized this inversion breakpoint near *BG:DS07295.3* (sequence coordinate 1746 kb). The two remaining inversion breakpoints are located one within P1 clone DS08284, which gave two hybridization signals in E4c-d and G3h, and the other one within P1 clone DS00941, which gave two signals also, one in G3h and the other one in E3c-d. In both cases, the hybridization signal at G3h was weaker compared with the other one. We propose that the inversion breakpoint within DS08284 is located in the 277–281-kb interval between the predicted genes *BG:DS00797.4* and

BG:DS00797.5 and that within DS00941 is located in the 328–344-kb interval between *RpII33* and *BG:DS00941.11*.

The estimated size of the six conserved chromosomal fragments varies from 50 to 531 kb (Table 1). The size of the outermost fragments is obviously a minimum estimate, since they can be extended for an unknown length outside the studied region. The average size is 324 kb (367 kb) with (without) the two outermost fragments, a much larger value than the expected size of the conserved chromosomal segment in chromosome 2, about 117 kb (Ranz *et al.* 1997, Ranz JM, Casals F, Ruiz A, in preparation). We can test the null hypothesis that the conservation of the 6 segments in this region is the result of the fixation of a finite number of chromosomal rearrangements with randomly located breakpoints (Nadeau & Taylor 1984, Ranz *et al.* 1997). If this hypothesis were rejected, then the operation of functional constraints could be invoked. The probability that a segment of relative length l is conserved (bears no breakpoint) between two species differing by n fixed inversions is $p = e^{-2nl}$. We have calculated this probability for each segment separately taking $n = 30$ as the number of fixed inversions (see above). The value of p was always ≥ 0.25. Thus there is no statistical evidence for functional constraints in any of the six segments. This result agrees with the sequence analysis of this region made by Ashburner *et al.* (1999) where they could not find any evidence of functional clustering of genes apart from the functional relatedness of duplicate genes, which simply reflects their common evolutionary origin.

The general conservation of a given chromosomal fragment does not preclude the occurrence of internal microrearrangements, which cannot be detected with the mapping technique used here. The 366-kb fragment which bears the *Adh* locus (see Table 1) is a good example. In *D. melanogaster*, the two loci *Adh* and *Adh-related* are arranged in tandem (Jeffs *et al.* 1994, Brogna & Ashburner 1997) and most probably originated with a gene duplication which occurred ~180 MYA, well before the radiation of the genus *Drosophila* (Russo *et al.* 1995). This explains their presence in the subgenera *Sophophora*, *Scaptodrosophila* and *Drosophila* (Marfany & González-Duarte 1991, Albalat & González-Duarte 1993, Juan *et al.* 1994, Russo *et al.* 1995, Amador & Juan 1999, Betrán & Ashburner, in preparation). Besides, species in the *mulleri* subgroup of the *repleta* group, including *D. buzzatti*, possess three *Adh* genes with the following transcriptional arrangement: *Adh-F*, *Adh-2* and *Adh-1* (Sullivan *et al.* 1994, Begun 1997). *Adh-F* is probably the result of an old duplication, which might predate the *repleta* radiation (~30 MYA; Throkmorton 1982). The other two genes, *Adh-2* and *Adh-1*, are the product of a more recent duplication (Sullivan *et al.* 1990, Menotti-Raymond *et al.* 1991, Russo *et al.* 1995). Therefore, in *D. buzzatii* at least (the organization in *D. repleta* is not known), the 'conserved' fragment bearing the *Adh* locus has suffered at least two different duplications after the divergence from the *D. melanogaster* lineage. Minor gene rearrangements in otherwise 'conserved' gene complexes have been reported in other cases as well (Randazzo *et al.* 1993).

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References

Adams MD, Celniker SE, Holt RA *et al.* (2000) The genome sequence of *Drosophila melanogaster*. *Science* **287**: 2185–2195.

Albalat R, González-Duarte R (1993) *Adh* and *Adh-dup* sequences of *Drosophila lebanonensis* and *D. immigrans*: interspecies comparisons. *Gene* **126**: 171–178.

Amador A, Juan E (1999) Non fixed duplication containing the *Adh* gene and a truncated form of the *Adhr* gene in the

Drosophila funebris species group: different modes of evolution of *Adh* relative to *Adhr* in *Drosophila*. *Mol Biol Evol* **16**: 1439–1456.

Ashburner M, Misra S, Roote J *et al.* (1999) An exploration of the sequence of a 2.9-Mb region of the genome of *Drosophila melanogaster*. The *Adh* region. *Genetics* **153**: 179–219.

BDGP (1999) Berkeley *Drosophila* Genome Project. Available at ⟨http://www.fruitfly.org⟩.

Begun DJ (1997) Origin and evolution of a new gene descendent from alcohol dehydrogenase in *Drosophila*. *Genetics* **145**: 375–382.

Betrán E, Santos M, Ruiz A (1998) Antagonistic pleiotropic effect of second-chromosome inversions on body size and early life-history traits in *Drosophila buzzatii*. *Evolution* **52**: 144–154.

Beverly SM, Wilson AC (1984) Molecular evolution in *Drosophila* and higher Dipterans. II. A time scale for fly evolution. *J Mol Evol* **21**: 1–13.

Brogna S, Ashburner M (1997) The Adh-related gene of *Drosophila melanogaster* is expressed as a functional dicistronic messenger RNA: Multigenic transcription in higher organisms. *EMBO J* **16**: 2023–2031.

Cáceres M, Ranz JM, Barbadilla A, Long M, Ruiz A (1999) Generation of a widespread *Drosophila* inversion by a transposable element. *Science* **285**: 415–418.

Cirera S, Martín-Campos JM, Segarra C, Aguadé M (1995) Molecular characterization of the breakpoints of an inversion fixed between *Drosophila melanogaster* and *Drosophila subobscura*. *Genetics* **139**: 321–326.

Grell EH, Jacobson KB, Murphy JB (1968) Alterations of genetic material for analysis of alcohol dehydrogenase isozymes of *Drosophila melanogaster*. *Ann NY Acad Sci* **151**: 441–455.

Hartl DL, Lozovskaya ER (1994) Genome evolution: between the nucleosome and the chromosome. In: Schierwater B, Streit B, Wagner GP, de Salle R, ed. *Molecular Ecology and Evolution: Approaches and Applications*. Basel: Birkäser, pp 579–592.

Hooper JE, Perez-Alonso M, Bermingham JR *et al.* (1992) Comparative studies of *Drosophila* Antennapedia genes. *Genetics* **132**: 453–469.

Jeffs PS, Holmes EC, Ashburner M (1994) The molecular evolution of the alcohol dehydrogenase and alcohol dehydrogenase-related genes in the *Drosophila melanogaster* species subgroup. *Mol Biol Evol* **11**: 287–304.

Juan E, Papaceit M, Quintana A (1994) Nucleotide sequence of the genomic region encompassing *Adh* and *Adh-dup* genes of *D. lebanonensis* (*Scaptodrosophila*): gene expression and evolutionary relationships. *J Mol Evol* **38**: 455–467.

Kress H (1993) The salivary gland chromosomes of *Drosophila virilis*: a cytological map, pattern of transcription and aspects of chromosome evolution. *Chromosoma* **102**: 734–742.

Labrador M, Naveira H, Fontdevila A (1990) Genetic mapping of the Adh locus in the repleta group of *Drosophila* by *in situ* hybridization. *J Hered* **81**(1): 83–86.

Lefevre G Jr (1976) A photographic representation and interpretation of the polytene chromosomes of *D. melanogaster* salivary glands. In: Ashburner M, Novitski E, eds. *The Genetics and Biology of Drosophila*, vol. 1a. London: Academic Press, pp 31–66.

Lemeunier F, Aulard S (1992) Inversion polymorphism in *Drosophila melanogaster*. In: Krimbas CB, Powell JR, eds. *Drosophila Inversion Polymorphism*. Boca Raton, Florida: CRC Press, pp 339–405.

Loukas M, Kafatos FC (1986) The actin loci in the genus *Drosophila*: establishment of chromosomal homologies among distantly related species by *in situ* hybridization. *Chromosoma* **94**: 297–308.

Maier D, Marte BM, Schafer W, Yu Y, Preiss A (1993) *Drosophila* evolution challenges postulated redundancy in the *E*(*spl*) gene complex. *Proc Natl Acad Sci USA* **90**: 5464–5468.

Marfany G, González-Duarte R (1991) The *Adh* genomic region of *Drosophila ambigua*: evolutionary trends in different species. *J Mol Evol* **32**: 454–462.

Martinez-Cruzado JC, Swimmer MG, Fenerjian MG, Kafatos FC (1988) Evolution of the autosomal chorion locus in *Drosophila*. I. General organization of the locus and sequence comparisons of genes *s15* and *s19* in evolutionary distant species. *Genetics* **119**: 663–677.

Menotti-Raymond M, Starmer WT, Sullivan DT (1991) Characterization of the structure and evolution of the *Adh* region of *Drosophila hydei*. *Genetics* **127**: 355–366.

Montgomery E, Charlesworth B, Langley CH (1987) A test for role of natural selection in the stabilization of transposable element copy number in a population of *Drosophila melanogaster*. *Genet Res* **49**: 31–41.

Muller JH (1940) Bearings of the *Drosophila* work on systematics. In: Huxley J, ed. *New Systematics*. Oxford: Clarendon Press, pp 185–268.

Nadeau JH, Taylor BA (1984) Lengths of chromosomal segments conserved since divergence of man and mouse. *Proc Natl Acad Sci USA* **81**: 814–818.

Nurminsky DI, Moriyama EN, Lozovskaya ER, Hartl DL (1996) Molecular phylogeny and genome evolution in the *Drosophila virilis* species group: duplication of the alcohol dehydrogenase gene. *Mol Biol Evol* **13**: 132–149.

Randazzo FM, Seeger MA, Huss CA, Sweeney MA, Cecil JK (1993) Structural changes in the Antennapedia complex of *Drosophila pseudoobscura*. *Genetics* **133**: 319–330.

Ranz JM, Segarra C, Ruiz A (1997) Chromosomal homology and molecular organization of Muller's element D and E in the *Drosophila repleta* species group. *Genetics* **145**: 281–295.

Ranz JM, Cáceres M, Ruiz A (1999) Comparative mapping of cosmids and gene clones from a 1.6 Mb chromosomal region of *D. melanogaster* in three species of the distantly related subgenus *Drosophila*. *Chromosoma* **108**: 32–43.

Ruiz A, Wasserman M (1993) Evolutionary cytogenetics of the *Drosophila buzzatii* species complex. *Heredity* **70**: 582–596.

Ruiz A, Ranz JM, Cáceres M, Segarra C, Navarro A, Barbadilla A (1997) Chromosomal evolution and comparative gene mapping in the *Drosophila repleta* species group. *Brazilian J Genet* **20**: 553–565.

Russo CAM, Takezaki N, Nei M (1995) Molecular phylogeny and divergence times of Drosophilid species. *Mol Biol Evol* **12**: 391–404.

Schafer DJ (1992) Developmental and molecular studies of *Adh* in *Drosophila buzzatii*. *Master Thesis*, University of New England Armidale, NSW, Australia.

Schafer DJ, Fredline DK, Knibb WR, Green MM, Barker JSF (1993) Genetics and linkage mapping of *Drosophila buzzatii*. *J Hered* **84**: 188–194.

Schneuwly S, Kuroiwa A, Gehring WJ (1987) Molecular analysis of the dominant homeotic Antennapedia phenotype. *EMBO J* **6**: 201–206.

Segarra C, Aguadé M (1992) Molecular organization of the X chromosome in different species of the *obscura* group of *Drosophila*. *Genetics* **130**: 513–521.

Segarra C, Lozovskaya ER, Ribó G, Aguadé M, Hartl DL (1995) P1 clones from *Drosophila melanogaster* as markers to study the chromosomal evolution of Muller's A element in two species of the *obscura* group of *Drosophila*. *Chromosoma* **104**: 129–136.

Sokal RR, Rohlf, FJ (1995) *Biometry*. New York: Freeman.

Stathakis DS, Pentz ES, Freeman ME (1995) The genetic and molecular organization of the dopa decarboxilase gene cluster of *Drosophila melanogaster*. *Genetics* **141**: 629–655.

Steineman UM (1982) Analysis of chromosomal homologies between two species of the subgenus *Sophophora: D. miranda* and *D. melanogaster* using cloned DNA segments. *Chromosoma* **87**: 77–88.

Steineman UM, Pinkser W, Sperlich D (1984) Chromosome homologies within the *Drosophila obscura* group probed by *in situ* hybridization. *Chromosoma* **91**: 46–53.

Sturtevant AH, Novitski E (1941) The homologies of the chromosome elements in the genus *Drosophila*. *Genetics* **26**: 517–541.

Sullivan DT, Atkinson PW, Starmer WT (1990) Molecular evolution of the alcohol dehydrogenase genes in the genus *Drosophila*. *Evolut Biol* **24**: 107–147.

Sullivan DT, Starmer WT, Curtiss SW, Menotti-Raymond M, Yum J (1994) Unusual molecular evolution of an *Adh* pseudogen in *Drosophila*. *Mol Biol Evol* **11**: 443–458.

Throckmorton LH (1982) The *virilis* species group. In: Ashburner M, Carson HL, Thompson JN eds. *The Genetics and Biology of Drosophila*, vol. 3b. New York: Academic Press, pp 227–296.

Vieira J, Vieira CP, Hartl DL, Lozovskaya ER (1997) A framework physical map of *D. virilis* based on P1 clones: applications in genome evolution. *Chromosoma* **106**: 99–107.

Von Allmen G, Hogga I, Spierer A *et al.* (1996) Splits in fruitfly *Hox* gene complexes. *Nature* **380**: 116.

Wasserman M (1992) Cytological evolution of the *Drosophila repleta* species group. In: Krimbas CB, Powell JR, eds. *Drosophila Inversion Polymorphism*. Boca Raton, Florida: CRC Press, pp 455–552.

Wharton LT (1942) Analysis of the *repleta* group of *Drosophila*. *Univ Texas Publ* **4228**: 23–52.