Restriction Map Variation at the *Adh* Locus of *Drosophila melanogaster* in Inverted and Noninverted Chromosomes

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ABSTRACT

Restriction map variation among 39 Standard and 40 *In(2L)t* chromosomes extracted from a Spanish natural population of *Drosophila melanogaster* was investigated for a 2.7-kb region encompassing the *Adh* locus with ten four-cutter restriction enzymes. A total of 20 polymorphisms were detected, representing 15 restriction site polymorphisms, 4 length polymorphisms and the allozyme polymorphism. Variation at the DNA level was compared among St-Adh^F, St-Adh^S and *t*-Adh^S chromosomes. *t*-Adh^S chromosomes show a higher level of variation than St-Adh^F chromosomes. This suggests that *In(2L)t* arose before the fast/slow allozyme divergence in the evolutionary history of *D. melanogaster*.

INVERSION polymorphisms in Drosophila are well documented at the descriptive level (DOBZHANSKY 1970; ASHBURNER and LEMEUNIER 1976; METTLER, VOELKER and MUKAI 1977; INOUE and WATANABE 1979; KNIBB 1982; KRIMBAS and LOUKAS 1980), but many questions concerning their origin and maintenance remain unanswered. Modern molecular techniques—Southern blot (SOUTHERN 1975), sequencing (MAXAM and GILBERT 1977; SANGER, NICKLEN and COULSON 1977) and four-cutter analysis (KREITMAN and AGUADÉ 1986a)—have enabled the study of variation at the nucleotide level at specific regions of the genome. These powerful techniques could be used to compare patterns of variation between standard and inverted chromosomes for loci in strong linkage disequilibrium with inversions. This information could provide some knowledge about the origin and relative age of inversions and could be considered a first effort at the molecular level to understand the evolution of inversions.

The *Adh* locus of *Drosophila melanogaster* has been chosen for the present study for the following reasons:

1. Adh^S shows a very strong linkage disequilibrium with inversion *In(2L)t* (KOJIMA, GILLESPIE and TOBARI 1970; MUKAI, METTLER and CHIGUSA 1971; LANGLEY, TOBARI and KOJIMA 1974; METTLER, VOELKER and MUKAI 1977; VOELKER *et al.* 1978; KNIBB 1982)—in fact, in population surveys the reported frequency of Adh^S in the inversion is either one or very close to one; *t* chromosomes are actually thought to be all Adh^S, those rare cases of alleged Adh^F-*t* chromsomes are considered to be actually *In(2L)Cy* chromosomes (a rare cosmopolitan inversion that has breakpoints only slightly different from *In(2L)t* and is known to be associated with Adh^F).

2. A wealth of knowledge has accumulated on the geographic distribution of variation both for *In(2L)t* and *Adh* allozymes (METTLER, VOELKER and MUKAI 1977; VOELKER *et al.* 1978; OAKESHOTT *et al.* 1982; KNIBB 1982).

3. The region including the *Adh* locus is the best documented in Drosophila as far as variation at the nucleotide level is concerned (LANGLEY, MONTGOMERY and QUATTLEBAUM 1982; KREITMAN 1983; BIRLEY 1984; KREITMAN and AGUADÉ 1986a, b; CROSS and BIRLEY 1986; AQUADRO *et al.* 1986 in *D. melanogaster* and BODMER and ASHBURNER 1984; COHN, THOMPSON and MOORE 1984; COYNE and KREITMAN 1986; SCHAEFFER, AQUADRO and ANDERSON 1987 in other species of Drosophila). There is only one report of variation at the DNA level within *In(2L)t* chromosomes, but the sample size for inverted chromosomes is very small (AQUADRO *et al.* 1986).

In this paper variation at the *Adh* structural locus is described for a rather large sample of both Standard and *t* chromosomes extracted from a single natural population previously analyzed both for inversion and enzyme polymorphism (AGUADÉ and SERRA 1980, 1987). In this population the frequency of Adh^S varies from 0.296 during vintage to 0.120 after overwintering; the frequency of *In(2L)t* in those two periods varies from 0.249 to 0.120. In this population, as in other European populations studied (AGUADÉ and SERRA 1980; AFONSO *et al.* 1985), the frequency of Adh^S in Standard chromosomes is very low—0.080 and 0.000 in the above-mentioned periods.

TABLE 1

Haplotypes and frequency distribution

Hap. no.	*Msp*I −340 1*	I0.17 −62 269 3*	*Ban*I 105 4*	*Hae*III 287	I1.4 448 6*	*Msp*I 502 505 8*	I1.40 551 9*	*Msp*I 586 11*	*Hae*III 816 14*	*Alu*I 1068 15*	*Ban*I 1307 1309	*Sau*3A 1354	*Sau*961 1425	F/S 1490 17*	*Dde*I 1518 18*	*Dde*I 1527 19*	*Alu*I 1596 22*	*Hae*III 1925 24*	*Taq*I 1987 1990	D3′.29 2379 2405	St/*t*	A80	A84	R80	Spain
1	+	−	−	+	−	+	−	+	−	+	+	+	+	F	+	−	+	+	+	−	St	1	1		2
2	+	−	−	+	+	+	+	+	+	+	−	+	+	F	+	−	+	+	+	−	St	1			1
3	+	−	−	+	+	+	+	+	+	+	+	+	+	F	+	−	+	+	+	−	St	7	12		19
4	+	−	−	+	−	−	−	+	−	+	+	+	+	F	+	−	+	+	+	−	St	1			1
5	+	−	−	+	+	+	+	+	−	+	+	+	+	F	+	−	+	+	+	−	St	2			2
6	+	−	−	+	+	+	−	+	−	+	+	+	+	F	+	−	+	+	+	−	St	1	3		4
7	+	−	−	+	−	+	−	+	+	+	+	+	+	F	+	−	+	+	+	−	St	1			1
8	+	−	−	+	−	+	+	+	+	+	+	+	+	F	+	−	+	+	+	−	St	1	2		3
9	+	−	+	+	+	+	−	+	−	+	+	+	+	F	+	−	+	+	+	−	St		1		1
10	+	−	−	+	+	+	+	−	+	+	+	+	+	F	+	−	+	+	+	−	St		1		1
11	−	+	−	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	−	St	1			1
12	+	−	+	+	−	+	−	+	−	+	+	+	+	S	+	−	−	+	+	+	St		1		1
13	+	−	+	+	−	+	−	+	+	+	+	+	+	S	+	−	+	+	+	−	St		1		1
14	+	−	+	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	−	St		1		1
15	−	+	−	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	−	*t*	2			2
16	+	−	+	+	−	+	−	+	−	+	+	+	+	S	+	−	−	+	+	+	*t*	2	5	1	8
17	+	−	+	+	−	+	−	+	+	+	+	+	+	S	+	−	+	+	+	−	*t*		1	1	2
18	+	−	+	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	−	*t*		3		3
19	+	−	+	+	−	+	−	+	−	+	+	−	+	S	+	−	+	+	+	−	*t*		2		2
20	+	−	+	+	−	+	−	+	+	+	+	−	+	S	+	−	+	+	+	−	*t*	1	5		6
21	−	−	−	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	−	−	*t*		2	1	3
22	+	−	+	−	−	+	−	+	−	−	+	−	−	S	−	+	+	+	+	+	*t*		3		3
23	−	−	+	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	−	*t*		2		2
24	+	−	−	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	−	−	*t*	1			1
25	−	−	−	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	+	*t*		1		1
26	−	−	+	+	−	+	−	+	−	+	+	+	+	S	−	+	−	−	+	−	*t*		1		1
27	+	+	+	+	−	+	−	+	−	+	+	−	+	S	+	−	+	+	+	−	*t*		1		1
28	+	−	+	+	−	+	−	+	−	+	+	+	+	S	−	+	−	−	+	+	*t*		1		1
29	+	−	+	+	−	+	−	+	−	+	+	+	+	S	+	−	−	+	+	−	*t*		1		1
30	+	+	−	+	−	+	−	+	−	+	+	+	+	S	+	−	+	+	+	−	*t*		1		1
31	+	−	−	+	−	+	−	+	−	−	+	+	+	S	+	−	+	−	+	−	*t*		1		1
32	+	−	+	+	−	+	−	+	−	+	+	+	+	S	−	+	+	−	+	−	*t*		1		1

+ and − show the presence or absence of restriction site and insertion or deletion variation. F, fast; S, slow. St, standard; *t*, *In(2L)t*. A80, Artés 1980; A84, Artés 1984; R80, Requena 1980. Numbers with an asterisk correspond to site numbers in KREITMAN and AGUADÉ (1986a). Positions are given according to KREITMAN (1983).

The analysis of variation at the *Adh* locus in inverted and noninverted chromosomes can provide valuable information concerning two major questions—the unique or multiple origin of *In(2L)t* and if unique, how old the inversion is.

MATERIALS AND METHODS

Drosophila stocks: Lines analyzed in the present study stem mainly from two different collections (October 1980 and October 1984) of the same cellar population of *D. melanogaster* (Artés, Spain). In both cases isofemale lines were established upon arrival in the laboratory, but they underwent a different treatment.

From the sample collected in 1980, lines homozygous for the left arm of the second chromosome were established by a controlled series of brother-sister crosses, using allozyme phenotype for α-Gpdh and Adh as markers (GRELL, JACOBSON and MURPHY 1965; GRELL 1967).

From the sample collected in 1984, isochromosomal lines were established by the Curly extraction procedure using *SM5/BIL*[2] as extractor stock (LINDSLEY and GRELL 1968). In order to avoid hybrid dysgenesis, a virgin female from each isofemale line was crossed to males from the extractor stock. The *Adh* genotype of each F_1 male was determined electrophoretically (GRELL, JACOBSON and MURPHY 1965) in order to increase the final sample size of isochromosomal lines homozygous for Adh^S.

Once second chromosome lines had been established, their karyotype for the left arm of the second chromosome was determined. Considering both Artés collections a total of 35 Standard Adh^F, 4 Standard Adh^S and 37 *In(2L)t* Adh^S chromosomes have been used in the present study.

Three additional lines homozygous for the *t* inversion and for Adh^S have been used. They were isolated from a sample collected in 1980 from a different Spanish cellar (Requena) and made homozygous by a controlled series of brother-sister crosses as in the case of the Artés 1980 collection.

Restriction map analysis: The procedure for preparing DNA and filters containing restriction digestions are described in KREITMAN and AGUADÉ (1986a). Ten restriction enzymes—*Alu*I, *Bam*HI, *Ban*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I, *Sau*3A, *Sau*96I and *Taq*I—were used in the present study. Filters were hybridized with a 2.7-kb gel-purified *Sal*I-*Cla*I fragment containing the *Adh* structural locus (KREITMAN 1983).

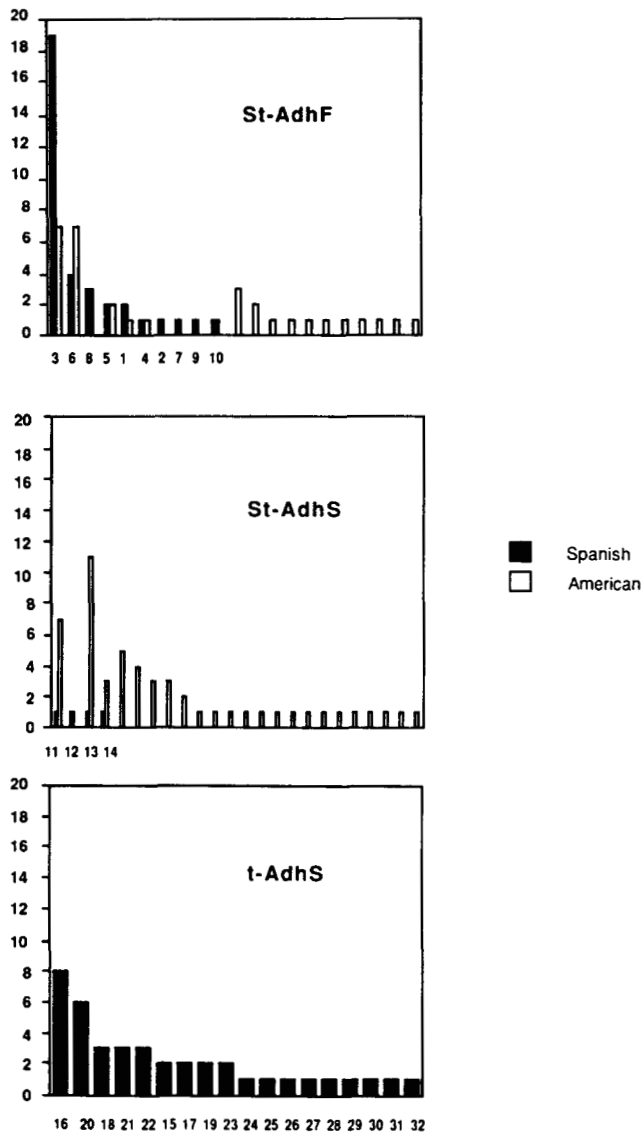

FIGURE 1.—Frequency distribution of haplotypes in the Spanish and North American samples under comparison (see text) sorted by allozyme and gene arrangement.

RESULTS

A total of 20 polymorphic sites were detected in the Spanish population analyzed, representing 15 restriction site polymorphisms, 4 length polymorphisms (3 insertions and 1 deletion) and the allozyme polymorphism. Table 1 shows the different haplotypes observed in the present study, sorted by gene arrangement—10 haplotypes for St-Adh^F, 4 haplotypes for St-Adh^S and 18 haplotypes for *t*-Adh^S chromosomes. Some of the insertions and deletions scored in North American populations (KREITMAN and AGUADÉ 1986a) have not been scored in the present study (sites 23, 25, 26 and 29 there). When compared to those North American populations, five new restriction site polymorphisms and one new deletion have been detected in the Spanish sample; this sample is monomorphic for seven restriction sites and for two insertions and two deletions which were segregating in North American samples.

Table 1 shows the frequency distribution of haplotypes in both samples of Artés and in Requena. The distribution of haplotypes within each gene arrangement is not significantly different between samples of Artés ($P > 0.25$ for both St and *t* chromosomes); neither does the distribution of haplotypes for *t* chromosomes in Artés (pooled sample) and Requena differ. The frequency distribution of haplotypes for the joint Spanish sample is also given in Table 1 and represented in Figure 1 and is the only one considered for further analysis. When gene arrangement and allozyme are not included as part

TABLE 2

***Adh* restriction map haplotype diversity**

Sample	n	Type of polymorphism: Restriction + Ins/Del	Restriction only	Ins/Del only
Spanish				
St-Adh^F	35	0.6941 ± 0.0816	0.4975 ± 0.0603	0.5939 ± 0.0876
St-Adh^S	4	1.0000 ± 0.1768	1.0000 ± 0.1768	0.8333 ± 0.2224
t-Adh^S	40	0.9282 ± 0.0209	0.9154 ± 0.0278	0.5679 ± 0.0538
American				
St-Adh^F	34	0.9109 ± 0.0291	0.8235 ± 0.0431	0.6410 ± 0.0980
St-Adh^S	53	0.9269 ± 0.0204	0.9158 ± 0.0437	0.5060 ± 0.0102

Haplotype diversity is calculated as $\hat{h} = n(1 - \sum x_i^2)/(n - 1)$ (NEI and TAJIMA 1981), where n is the number of chromosomes sampled and x_i is the frequency of the ith haplotype; this measure is not only influenced by the number of haplotypes observed but by their frequencies in the sample.

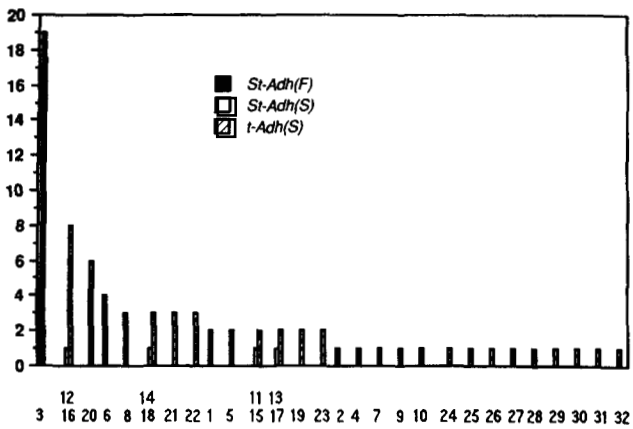

FIGURE 2.—Frequency distribution of haplotypes in the Spanish sample.

of the haplotype, there is no overlapping between haplotypes present in St-Adh^F lines and those present in St-Adh^S or t-Adh^S lines; however, the four haplotypes present in St-Adh^S lines are also present in the t lineage (Figure 2).

Figure 1 shows the frequency distribution of haplotypes both for the Spanish and the North American samples under comparison (KREITMAN and AGUADÉ 1986a). The main difference between Spanish and North American populations is the complete absence of inversion $In(2L)t$ in the North American samples considered in this study (M. Aguadé, unpublished result). 5 out of 10 different haplotypes present in Spanish St-Adh^F chromosomes and 3 out of 4 present in Spanish St-Adh^S chromosomes are also present in North America.

Restriction map heterozygosity has been used to measure haplotype diversity (NEI and TAJIMA 1981). Heterozygosity has been calculated separately for St-Adh^F, St-Adh^S and t-Adh^S chromosomes (Table 2). St-Adh^S chromosomes show the same level of variation as t chromosomes both when restriction site only and when all variation is considered. Standard fast chromosomes are significantly less variable than slow chromosomes (either St or t) when restriction site variation is considered, but they do not differ significantly from t chromosomes for insertion/deletion variation. When haplotype diversity is considered, Spanish St-Adh^F chromosomes show a lower level of variation for restriction site polymorphism than American St-Adh^F chromosomes but do not differ significantly for insertion/deletion variation; Spanish t-Adh^S chromosomes show the same level of variation for both restriction site and insertion/deletion variation than American St-Adh^S chromosomes.

TABLE 3

Heterozygosity per nucleotide

Samples	n	
Spanish		
St-Adh^F	35	0.00091 ± 0.00018
St-Adh^S	4	0.00282 ± 0.00082
t-Adh^S	40	0.00461 ± 0.00025
American		
St-Adh^F	34	0.00289 ± 0.00029
St-Adh^S	53	0.00567 ± 0.00029

When heterozygosity per nucleotide is estimated (ENGELS 1981) for St-Adh^F, St-Adh^S and t-Adh^S chromosomes (Table 3), St-Adh^F chromosomes are significantly less variable than inverted chromosomes. When nucleotide heterozygosity in the Spanish sample is compared to that in North American populations, Spanish St-Adh^F chromosomes are significantly less variable than American St-Adh^F chromosomes (3.18 times less variable); Spanish t-Adh^S chromosomes are significantly less variable than American St-Adh^S chromosomes (1.23 times less variable) but at the same time significantly more variable than American St-Adh^F chromosomes (1.60 times more variable).

DISCUSSION

Because of the low probability that inversions are generated more than once with exactly the same break points, it is generally thought that naturally occurring inversions have unique evolutionary origins (DOBZHANSKY 1970). The pervasive existence in different Drosophila species of strong linkage disequilibria between some allozyme loci and inversions has been a strong argument against multiple origins of inversions. The finding of transposable elements in Drosophila raised speculation as to the possibility of their playing some role in the origin of inversions. If inversions are caused by the activities of transposable elements, multiple origins of cytologically indistinguishable inversions might exist. Although it is known that breakpoints of chromosome rearrangements induced by the activity of the *P* element family in *D. melanogaster* occur at or very near the sites of preexisting *P* elements (ENGELS and PRESTINE 1984) and although there is some evidence for an excess of transposable elements near the breakpoints of artificial inversions in balancer stocks (MONTGOMERY, CHARLESWORTH and LANGLEY 1987), little is known, in precise molecular terms, about naturally occurring inversions. The question about the origin of inversions remains unanswered, awaiting for some knowledge about the molecular biology of inversion break points.

An indirect approach to study the origin and evolution of inversions is to examine the divergence of sequences that are in strong linkage disequilibrium with an inversion. If a particular gene arrangement had a unique origin, one would expect a positive correlation between the age and the amount of variation harbored by that gene arrangement.

AQUADRO *et al.* (1986) reported two different haplotypes in a sample of five *In(2L)t* lines, one of the haplotypes being explained either by recombination between a fast (never *In(2L)t*) and a slow sequence that was *In(2L)t* or by a multiple origin of *In(2L)t*. They concluded that the strong association of *Adh* allozyme and DNA sequence variants with *In(2L)t* probably reflects evolutionary history and a relatively recent origin of that inversion, although they suggested that the study of a bigger sample is needed.

In the Spanish sample there are eight polymorphic sites unique to the inversion. There are however six shared polymorphisms between the noninverted and inverted chromosomes (essentially between St-Adh^S and *t*-Adh^S chromosomes). The existence of shared polymorphisms does not allow to discriminate between a unique and a multiple origin of inversion *In(2L)t*. In case of a monophyletic origin of the inversion, those shared polymorphisms could be explained either by rare recombination or gene conversion between both lineages or by a high mutation rate at some sites. The fact that all inverted chromosomes are Adh^S is still the only strong evidence indicating that inversion *In(2L)t* has not arisen many times.

Previous population surveys of restriction site polymorphisms (BIRLEY 1984; KREITMAN and AGUADÉ 1986a; CROSS and BIRLEY 1986; AQUADRO *et al.* 1986) have provided evidence for a lower level of variation in fast chromosomes as compared to slow chromosomes, a result consistent with a more recent ancestry for the Adh^F allele. In the Spanish sample St-Adh^F chromosomes show a significantly lower level of variation (estimated either as haplotype diversity or heterozygosity per nucleotide) than both St-Adh^S and *t*-Adh^S lines. One could argue that Spanish St-Adh^F chromosomes do show a very low level of variation when compared to North American St-Adh^F lines and that decreased variability might be biasing the comparison in the Spanish sample. When *t*-Adh^S chromosomes from Spain are compared to St-Adh^F and St-Adh^S chromosomes from North America, the pattern observed in the Spanish sample does hold—*t*-Adh^S chromosomes are more variable than American St-Adh^F chromosomes (heterozygosity per nucleotide is in this case 1.60 times higher as opposed to 5.07 times higher in the Spanish sample) and slightly less variable than American St-Adh^S chromosomes (heterozygosity is 1.23 times lower; in the Spanish sample St-Adh^S chromosomes do in fact show lower heterozygosity than *t*-Adh^S chromosomes, probably due to the small sample size). This result would suggest that if *In(2L)t* has a unique origin from an Adh^S carrying chromosome, this event should have happened a long time ago, in order for the unique sequence captured by the inversion event to have had time not only to accumulate variation but also to have nearly reached equilibrium as compared to the Adh^S Standard lineage. Comparison with fast chromosomes indicates that the *t* inversion event should have happened long before the fast/slow allozyme divergence or at least before the actual divergence from the slow lineage of the single progenitor of all extant fast sequences (AQUADRO *et al.* 1986). An alternative would be that *In(2L)t* originated more than once, but then an explanation is needed for its recurrence and mainly for the strong linkage disequilibrium with Adh^S allozyme. The observation that haplotype diversity due to length polymorphism has reached the same level in St-Adh^F, St-Adh^S and *t*-Adh^S is in agreement with previous studies (KREITMAN and AGUADÉ 1986a; AQUADRO *et al.* 1986) and therefore with the suggestion that this kind of variation occurs at a higher rate than base substitution reaching equilibrium faster.

In this initial descriptive phase it would be important to study large samples of *In(2L)t* chromosomes from different geographical areas in order to assess

possible geographic patterns of inversion differentiation and to get further information on the age, expansion and evolution of the inversion and at the same time of the species.

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