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The evolutionary history of *Drosophila buzzatii*. XXXII. Linkage disequilibrium between allozymes and chromosome inversions in two colonizing populations

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Chromosome polymorphism in *Drosophila buzzatii* is under selection but the genes responsible for the effect of the inversions on fitness are unknown. On the other hand, there is evidence for selection on several allozyme loci but the presence of paracentric inversions on the second chromosome, where most of the polymorphic loci are located, complicates the interpretation. Studies of the associations between allozymes and inversions are thus necessary to help understand the effect of selection at both the chromosomal and allozymic level. Until now this kind of information has only been available in *D. buzzatii* for two loci, *Est-1* and *Est-2*, in Australian populations. Here we describe the genetic constitution of two Old World populations, Carboneras and Colera. Emphasis has been placed on the analysis of the linkage disequilibria between the second chromosome arrangements and three allozyme loci, *Est-2, Pept-2* and *Aldox*, located on this chromosome. In addition, the recombination frequencies between the loci, and between the loci and the inversion breakpoints, have been estimated and a genetic map of the three loci has been produced. The two populations differ in allele and arrangement frequencies, as well as in the pattern of one-locus disequilibria. *Est-2* and *Aldox* are associated with the second chromosome arrangements in both populations. On the other hand, *Pept-2* is associated with the inversions in Colera but not in Carboneras. The gametic associations among the three loci are discussed taking into account the position of these loci on the chromosome map and the lack of recombination in the heterokaryotypes.

Keywords: allozymes, cactophilic *Drosophila*, chromosome inversions, colonization, *Drosophila buzzatii*, linkage disequilibrium.

Introduction

The colonizing species *Drosophila buzzatii* (repleta group, *buzzatii* complex; Ruiz & Wasserman, 1993) originated in South America but has spread within historical times to the Canary Islands, the Mediterranean region, Australia and several other places throughout the world (David & Tsacas, 1980; Barker *et al.*, 1985; Fontdevila, 1989). It is specific to the cactus niche and uses the decaying stems of several species of the genera *Opuntia, Cereus* and *Trichocereus* as feeding and breeding substrates (Fontdevila, 1981; Pereira *et al.*, 1983; Hasson *et al.*, 1992).

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The karyotype of *D. buzzatii* consists of six pairs of chromosomes: four pairs of equal length acrocentric autosomes, one pair of dot chromosomes, a long acrocentric X and a small acrocentric Y chromosome (Wasserman, 1962). The second and fourth chromosomes are polymorphic for a total of eight paracentric inversions (Ruiz *et al.*, 1984; Ruiz & Wasserman, 1993). All the polymorphic inversions are present in South America (Fontdevila *et al.*, 1982; Ruiz *et al.*, 1984) whereas in the Old World populations only six arrangements (2st, 2j, $2jz^3$, $2jq^7$, 4st and 4s), have been recorded (Fontdevila *et al.*, 1981; Fontdevila, 1989). All these arrangements, except $2jq^7$ and 4s, are also present in Australia (Knibb *et al.*, 1987).

Several lines of research have provided evidence for an adaptive role of the inversion polymorphisms in *D.*

buzzatii. (1) Experimental populations subject to different nutritional regimes exhibit rapid and consistent changes in arrangement frequencies (Ruiz & Fontdevila, 1985; Ruiz *et al.*, 1987). (2) Latitudinal clines in the frequencies of some inversions have been observed both in the original and colonized areas (Ruiz, 1982; Knibb *et al.*, 1987). (3) An analysis of selection components carried out in Carboneras (Spain) indicated viability differences between the second chromosome karyotypes (Ruiz *et al.*, 1986; Santos *et al.*, 1989). (4) The second chromosome karyotypes have an effect, in both the field and the laboratory, on body size (Ruiz *et al.*, 1991; Hasson *et al.*, 1992), an adaptive trait which shows a positive correlation with adult fitness components in natural populations (Santos *et al.*, 1988, 1992; Ruiz & Santos, 1989). The effect of the karyotype on body size and fitness is thought to be caused by the association of different arrangements with particular alleles that influence these traits (Ruiz *et al.*, 1991). The genes responsible, however, have not yet been identified.

The second chromosome, which carries the majority of rearrangements in *D. buzzatii*, also exhibits much greater allozyme variability than the remaining chromosomes (Barker, 1981; Quezada, 1993). Some studies have revealed that selection acts also on some of these allozyme loci. Perturbation experiments (Barker & East, 1980; Barker *et al.*, 1989), long-term temporal studies on variation in allele frequencies over time and correlation with environmental variables (Barker, 1981; Barker *et al.*, 1986), studies of autocorrelation (Sokal *et al.*, 1987), and experiments of heat and cold-shock resistance (Watt, 1981) suggest an adaptive value for the variation at the *Est-1, Est-2, Pyr, Adh-1* and *Aldox* loci. In all these studies, the presence of polymorphic inversions on the second chromosome complicates interpretation because the detected allozyme frequency changes could result from selection acting in some way on the inversions rather than on the allozyme loci. It is thus clear that studies of linkage disequilibrium between allozymes and inversions are necessary to understand the adaptive significance of allozyme variability in *D. buzzatii*. Until now only one such study has been undertaken. Knibb *et al.* (1987) described the associations between *Est-1* and *Est-2* and the second chromosome arrangements in several Australian populations but no information is available for the Old World populations.

We describe here the inversion and allozyme polymorphisms in two populations of the Iberian Peninsula and the linkage disequilibria between the second chromosome arrangements and three allozyme loci, *Est-2, Pept-2* and *Aldox*, located on this chromosome. Only limited information on the location and genetic distances among these genes is currently available (Schafer *et al.*, 1993). Therefore, a genetic map of the three loci has been obtained and the recombination frequencies between the loci, and between each locus and the inversion breakpoints, have been estimated. When analysing the linkage disequilibrium between loci linked to inversions, the absence of recombination in the heterokaryotypes must be taken into account (Zouros & Krimbas, 1973). Thus, we partitioned the total disequilibrium between two loci into different components: *within* and *between* chromosome arrangements (A. Navarro *et al.*, unpublished data). The genetic constitution of the two Old World populations is discussed in relation to colonization.

Materials and methods

Natural populations studied and processing of the samples

Two colonized populations of *Drosophila buzzatii* in the Old World, Carboneras (37° N 1.9° W) and Colera (42.37° N 3.16° E), were sampled. Both are located on the Mediterranean coast of the Iberian Peninsula but differ in several respects. The collecting site in Carboneras (Almería, S.E. Spain) is an abandoned *Opuntia ficus-indica* (prickly pear) plantation situated in a hot and dry region (see Ruiz *et al.*, 1986 for a detailed description). The genetic constitution and structure of this population has been previously studied in detail (Fontdevila *et al.*, 1981; Ruiz *et al.*, 1986; Santos *et al.*, 1989; Quezada-Díaz *et al.*, 1992; Barbadilla *et al.*, 1994). Although there are large fluctuations in the number of adults over the seasons, the population is without any doubt a permanent one and the effective population number is likely to be high (Santos *et al.*, 1989).

Colera (Girona, N.E. Spain), on the other hand, is located in a torrent on the northern Mediterranean coast. A cactus species, tentatively identified as *Opuntia dillenii* (Britton & Rose, 1963; Backeberg, 1977), grows along a dry river bed and is scattered over a strip about 25 m wide and 1 km long. A few *O. ficus-indica* plants are also present on the nearby hillsides. *D. buzzatii* larvae have been observed in rotting *O. dillenii* fruits. The region is quite humid (with an average rainfall of 1000 mm per year). However, the general vegetation is low because of the strong and cold wind coming from the sea. Winter is relatively cold (the average temperature during January is 8°C). In July 1991, 5 days of intensive collecting with banana baits and aspirating from *Opuntia* rots yielded 601 adults. In a second collection next autumn (31 October and 5 November 1991), only 10 females and

20 males could be caught. Thus, the size of this population is small and suffers a marked reduction during the autumn–winter season. We do not know at the moment whether the population is permanent or is recolonized every spring from refugia in the nearby area.

Over 1000 adults were collected in Carboneras between 14 July and 16 July 1990 by means of banana baits scattered throughout the sampling area. Males were individually crossed with 4–5-day-old virgin females from a *D. buzzatii* stock fixed for inversion 2j and alleles *Est-2*^{a} and *Pept-2*^{b} (see below). One third-instar larva from the F_1 of each cross was dissected in saline solution and the salivary gland chromosomes prepared as described in Fontdevila *et al.* (1981). Following Knibb *et al.* (1987), we also analysed electrophoretically the genotypes at the *Est-2* and *Pept-2* loci of the same larvae (using the rest of the body). In this way, we determined, in a sample of gametes, the arrangements present on the second chromosome as well as the alleles at the *Est-2* and *Pept-2* loci. In addition, wild-caught males were genotyped for *Aldox, Adh-1, Pept-2* and *Est-2* by means of electrophoresis.

The population of Colera was sampled from 16 July to 19 July 1991. All the adults, 365 males and 236 females, were individually crossed with virgin flies of the opposite sex from a stock fixed for arrangement 2jz^3 and alleles *Est-2*^{d} and *Pept-2*^{b}. Wild females were kept in mass cultures for a week to allow them to lay any fertilized eggs that they might have been carrying. In addition, the cross females were transferred every 2 days to new vials with fresh food, and the first vial of each cross was discarded altogether. This experimental procedure, because of sperm predominance in *D. buzzatii* (Barbadilla *et al.*, 1991), reduces the possibility of wild-fathered larvae in the analysed sample. Allele *Est-2*^{d}, missing in the population of Colera, provided a further test for the correct paternity of the analysed larvae. F_1 larvae were analysed as above to determine the gametic frequencies for the second chromosome, and the *Est-2* and *Pept-2* loci. Gametic disequilibria between *Pept-2, Est-2* and *Aldox* were studied using F_1 adults because aldehydo-oxidase only shows enough activity in the adult stage. In addition, wild adults were genotyped for *Est-2, Pept-2* and *Adh-1*.

Allozyme electrophoresis

Four allozymic loci, *Est-2, Pept-2, Aldox* and *Adh-1* that encode for soluble enzymes, were analysed by horizontal starch gel electrophoresis (see Quezada-Díaz *et al.*, 1992, for details of electrophoretic techniques). The four loci have been found previously to be polymorphic in Carboneras (Quezada-Díaz *et al.*, 1992). Recently, five alleles have been detected for *Est-2* in Carboneras (*Est-2*^{a}, *Est-2*^{b}, *Est-2*$^{c+}$, *Est-2*^{c} and *Est-2*^{d}) and all except *Est-2*^{d} in Colera. Under our electrophoretic conditions, alleles *Est-2*^{b} and *Est-2*$^{c+}$ show very close bands in the zymograms. Therefore these two alleles were not distinguished in our previous work (under *Est-2*^{b} in Quezada-Díaz *et al.*, 1992) and in some of the samples here (under *Est-2*^{b*}).

Genetic map of the second chromosome

Est-2, Aldox and *Pept-2* are located on the second chromosome (Schafer *et al.*, 1993; this work) while *Adh-1* has been mapped to the third chromosome (Labrador *et al.*, 1990). The three loci located on the second chromosome were mapped using *D. buzzatii* stocks fixed for different arrangements and allele combinations. Three-point tests were carried out to determine the linear relationships and the relative distances among the three loci both in the 2st and 2j gene arrangments. The distances from *Est-2* and *Pept-2* to the breakpoints of inversions 2j and 2q^7 were also estimated. Recombination frequencies and their standard errors were obtained following Elandt-Johnson (1971, pp. 427–431). Because we are most interested in the consequences at the population level, recombination frequencies were taken directly as estimates of genetic distance without transformation by means of a mapping function.

Data analysis

Deviations from Hardy–Weinberg expectations were measured using the one-locus disequilibrium coefficients ($D_A{}^{ij}$), and each statistically independent coefficient was tested with a χ^2 statistic (Weir, 1990, pp. 71–89). For a multiallelic locus, the departure of the total number of heterozygotes from the expected number can also be tested by a $\chi^2{}_1$ test, although this is a conservative procedure (Pamilo & Varvio-Aho, 1984). Gametic disequilibrium was measured using the D coefficient of Lewontin & Kojima (1960). For multiallelic loci, each statistically independent coefficient was tested using the χ^2 statistic (Weir, 1990, p. 93). The overall hypothesis that none of the statistically independent coefficients is different from zero was tested using the χ^2_T test (Weir, 1990, p. 94), which is equivalent to a contingency table.

The analysis of the gametic disequilibrium between two loci, A and B, is complicated when they are linked to polymorphic chromosome inversions. The total disequilibrium between A and B can be partitioned into 'within arrangements' and 'between arrangements' components (A. Ruiz *et al.*, 1991 and unpublished

data). In the particular case of a population polymorphic for four chromosome arrangements, as Carboneras and Colera, this partition is:

$$D^{ij}_{AB} =$$
$$u_1 D^{ij}_{AB(C_1)} + u_2 D^{ij}_{AB(C_2)} + u_3 D^{ij}_{AB(C_3)} + u_4 D^{ij}_{AB(C_4)}$$
$$+ \frac{D^{i}_{AC_1} D^{j}_{BC_1}}{u_1} + \frac{D^{i}_{AC_2} D^{j}_{BC_2}}{u_2} + \frac{D^{i}_{AC_3} D^{j}_{BC_3}}{u_3} + \frac{D^{i}_{AC_4} D^{j}_{BC_4}}{u_4},$$

where u_k ($k = 1, \ldots, 4$) is the frequency of the arrangement C_k; $D^{ij}_{AB(C_k)}$ is the disequilibrium coefficient between the ith and the jth alleles of loci A and B, respectively, within arrangement C_k; and $D^{i}_{AC_k}$ ($D^{j}_{BC_k}$) is the disequilibrium between allele i (j) of locus A (B) and the gene arrangement C_k. This partition of the total disequilibrium is necessary for interpreting the origin of the disequilibria because in a random mating population each component decays at a different rate (A. Navarro *et al.*, unpublished data). In general, the *within* arrangements disequilibria decay at a faster rate than the *between* arrangements disequilibria. Therefore, stronger associations are expected between allozymes and inversions than between allozymes within chromosome arrangements.

Three-locus disequilibria as defined by Bennet (1954) were also estimated in some cases. Relative disequilibria (D') were calculated following Lewontin (1964) for the pair-wise disequilibria, and following Robinson *et al.* (1991) for the three-locus disequilibria. Weir (1990) provides a way of testing the three-locus disequilibria (note that the square root in his formula on p. 96 for the corresponding χ^2 statistic must be misprinted, c.f. with Appendix C, p. 306).

All data analyses were carried out using the CSS-Statistica package run on a PC-compatible or using the BMDP Statistical Software run on a Vax-6610 VMS at the Centre de Càlcul of the Universitat Autònoma de Barcelona. Three-locus disequilibria were calculated and tested using computer program TLD written in Q BASIC by J. M. Ranz.

Results

Genetic map of the Est-2, Aldox *and* Pept-2 *loci*

Figure 1 summarizes the information obtained for the genetic map. *Est-2* and *Pept-2* were found to be the most distant markers, on both the 2st (RF = 50.5 ± 6.8) and the 2j chromosomes (RF = 46.5 ± 5.8), and to segregate independently. *Aldox* is located in between on both the 2st and 2j gene arrangements. In addition, *Est-2* must be close to the proximal breakpoint of inversion 2j and within the $2q^7$ region as no recombinants were detected with either inversion. On the other hand, *Pept-2* must be located on a distal position as recombinants between *Pept-2* and inversions 2j and $2q^7$ were observed.

Gene and genotypic frequencies and one-locus disequilibria

Table 1 gives the arrangement frequencies obtained from the gametes derived from wild-caught flies. At Carboneras, the frequencies were similar to those reported in earlier collections (Fontdevila *et al.*, 1981; Ruiz *et al.*, 1986, 1991). Although the same polymorphic inversions are present in both populations,

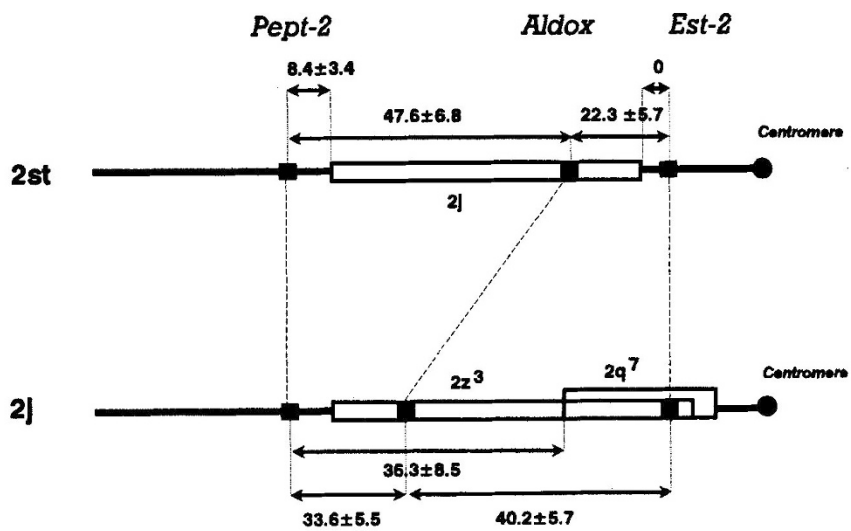

Fig. 1 Genetic map on arrangements 2st and 2j of loci *Aldox*, *Pept-2* and *Est-2* in the species *Drosophila buzzatii*. Frequencies of recombination (RF) and their 95 per cent confidence intervals are given.

Table 1 Arrangement frequencies for the second and fourth chromosomes in two samples of *Drosophila buzzatii* collected in Carboneras and Colera (Spain)

	Carboneras	Colera		χ^2 for differences between	
Arrangement	Males	Males	Females	Sexes	Populations
2st	0.4289	0.1311	0.1649	1.90	103.03***
2j	0.3807	0.4909	0.4433	(d.f. = 3)	(d.f. = 3)
$2jz^3$	0.1548	0.2652	0.2887		
$2jq^7$	0.0356	0.1128	0.1031		
N	394	328	194		
4st	0.7584	0.5640	0.6082	0.98	31.27***
4s	0.2416	0.4360	0.3917	(d.f. = 1)	(d.f. = 1)
N	389	328	194		

***$P \leq 0.001$.

noticeably lower frequencies of the standard arrangements (2st and 4st) were found in Colera. No differences between sexes were detected at Colera.

Allele frequencies for *Aldox, Adh-1, Pept-2* and *Est-2* in Carboneras and Colera are given in Table 2. All the data in this table come directly from the genotypes of wild adults, except the allele frequencies for *Aldox* in Colera which come from the sample of gametes. Only *Adh-1* shows significantly different frequencies between the sexes in Colera. When allele frequencies were compared between Carboneras and Colera all tests yielded highly significant results. Alleles $Aldox^a$, $Adh\text{-}1^b$, $Pept\text{-}2^a$, $Est\text{-}2^{b}$* and $Est\text{-}2^c$ have higher frequencies in Colera than in Carboneras.

In Carboneras, there was an excess of heterozygotes (negative D_A values) at all four loci, but only that for *Est-2* was significant (Table 2). By contrast, the majority of D_A values in Colera were positive, i.e. homozygotes were in excess, and those for *Est-2* in males and females and that for *Adh-1* in males were significant (Table 2).

Gametic frequencies and linkage disequilibria

Gametic frequencies for *Est-2, Pept-2* and the second chromosome arrangements in Carboneras and Colera reflect departure from linkage equilibrium in both populations: out of 32 possible haplotypes only 14 were present in Carboneras and 10 in Colera. Contingency χ^2 tests for the three pair-wise combinations gave the following results: *Est-2* alleles are strongly associated with the second chromosome arrangements in both populations (Carboneras: $\chi^2_9 = 431.82$; Colera: males $\chi^2_9 = 881.92$; females $\chi^2_9 = 539.97$; $P < 0.001$ in all three comparisons); *Pept-2* was associated with the second chromosome arrangements in the population of Colera but not in Carboneras (Carboneras: $\chi^2_3 = 0.43$ NS; Colera: males $\chi^2_3 = 28.14$; females $\chi^2_3 = 13.50$ and $P < 0.001$ in both sexes). Finally, *Est-2* and *Pept-2* were significantly associated in both populations (Carboneras: $\chi^2_3 = 9.01$, $P < 0.05$; Colera: males $\chi^2_3 = 23.32$, $P < 0.001$; females $\chi^2_3 = 8.92$, $P < 0.01$). Results of the partition of the disequilibria *within* and *between* inversions are given in Table 3. The analysis was carried out separately for each population and sex because, owing to the lack of recombination in *Drosophila* males, haplotype frequencies in the gametes produced by the two sexes may differ. In this table four values for each disequilibrium parameter, corresponding to the four *Est-2* alleles, are given.

Within arrangement disequilibria had, in general, very low and nonsignificant values. The only single exception was the disequilibrium within arrangement $2jz^3$, $D_{AB(2jz3)}$, in the Carboneras sample with the combination of alleles $Est\text{-}2^c$ and $Pept\text{-}2^a$ in excess. Disequilibria between *Est-2* alleles and second chromosome arrangements were highly significant in both populations but, comparing the D' values, Colera shows even stronger disequilibria than Carboneras. The 2st arrangement contains two alleles, $Est\text{-}2^a$ (in excess) and $Est\text{-}2^{b}$*, in the population of Carboneras, but only one, $Est\text{-}2^a$, in Colera. On the other hand, the 2j arrangement holds both alleles in both populations but with $Est\text{-}2^{b}$* in excess and $Est\text{-}2^a$ in deficit. The $2jz^3$ arrangement harbours alleles $Est\text{-}2^c$ and $Est\text{-}2^d$ in Carboneras and only $Est\text{-}2^c$ in Colera. Finally, in Colera the $2jq^7$ arrangement is fully associated with allele $Est\text{-}2^{c+}$. In Carboneras this arrangement was found to contain the $Est\text{-}2^{b}$* allele which, as noted

Table 2 Allele frequencies of *Aldox, Adh-1, Pept-2* and *Est-2*, in two samples of wild *Drosophila buzzatii* adults collected in Carboneras and Colera (Spain). Intralocus disequilibrium coefficients (D) and sample sizes of individuals (N) are also given

		Carboneras	Colera		χ^2 for differences between	
Locus	Allele†	Males	Males	Females	Sexes	Populations
Aldox	*a*	0.6951	0.8613			25.04***
	b	0.3049	0.1387			(d.f. = 1)
	D	−0.014				
	N	328	238			
Adh-1	*b*	0.5887	0.8090	0.8719	4.72*	71.82***
	c	0.4113	0.1910	0.1281	(d.f. = 1)	(d.f. = 1)
	D	−0.011	0.026**	0.017		
	N	355	288	121		
Pept-2	*a*	0.4465	0.4971	0.4954	0.00	4.50*
	b	0.5535	0.5029	0.5046	(d.f. = 1)	(d.f. = 1)
	D	−0.002	−0.024	0.009		
	N	374	345	220		
Est-2	*a*	0.5818	0.2098	0.2296	4.36	229.19***
	*b**	0.2961	0.5045	0.4975	(d.f. = 2)	(d.f. = 3)
	c	0.0896	0.2857	0.2729		
	d	0.0325	—	—		
	D_T	−0.036***	0.036**	0.030**		
	D_{ab*}	−0.030**	0.028**	0.033*		
	D_{ac}	−0.015***	0.005	−0.019		
	D_{ad}	−0.010**	—	—		
	D_{b*c}	0.010*	0.003	0.016		
	D_{b*d}	0.006*	—	—		
	D_{cd}	0.003	—	—		
	N	385	336	196		

For *Aldox* Colera males, N is the number of gametes.
†Allele *b** of the *Est-2* locus stands for alleles *b* and *c*+ pooled (see text).
*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

above, results from pooling *Est-2*^{b} and *Est-2*$^{c+}$. A recent analysis of six 2st and six 2j isochromosomal lines extracted from Carboneras has shown that none of them contains the *Est-2*$^{c+}$ allele, whereas six 2jq^7 lines from the same population hold only this allele. Therefore, the total association between the 2jq^7 arrangement and the *Est-2*$^{c+}$ allele observed in Colera is very likely to be the same in both populations. Disequilibria between *Pept-2* and the second chromosome arrangements were nonsignificant in Carboneras but highly significant in Colera, where *Pept-2*^{a} was associated with the 2st arrangement in both sexes.

In Carboneras, total disequilibria between *Est-2* and *Pept-2* were in general low and nonsignificant, as expected from the lack of disequilibria within arrangements and the lack of association between *Pept-2* and the second chromosome arrangements. Only the combination between alleles *Est-2*^{d} and *Pept-2*^{a} exhibited a significant deficit chiefly resulting from the disequilibrium shown by this combination within the 2jz^3 arrangement. In Colera, two of the four disequilibria between *Est-2* and *Pept-2* in males were significant: *Pept-2*^{a} was found in excess in combination with *Est-2*^{a}, and in deficiency when combined with *Est-2*^{c}. The former allele combination showed a significant excess also in females. These total disequilibria result chiefly from the between arrangements component and match closely the pattern of associations observed between *Pept-2* and the chromosome inversions because the association between *Est-2* and the inver-

Table 3 Estimates for *Drosophila buzzatii* of gametic disequilibrium (D) for the various combinations between the four *Est-2* alleles (locus *A*), *Pept-2*^{a} (locus *B*) and the four second chromosome arrangements from 344 gametes of wild males collected at Carboneras, 328 gametes of wild males and 194 gametes of wild females collected at Colera

	Carboneras				Colera males				Colera females			
D	*Est-2*^{a}	*Est-2*^{b}*	*Est-2*^{c}	*Est-2*^{d}	*Est-2*^{a}	*Est-2*^{b}	*Est-2*$^{c+}$	*Est-2*^{c}	*Est-2*^{a}	*Est-2*^{b}	*Est-2*$^{c+}$	*Est-2*^{c}
$D_{AB(2st)}$	0.0248	−0.0248	—	—	0.0000	—	—	—	0.0000	—	—	—
	(0.19)	(−0.19)	—	—	(0.00)	—	—	—	(0.00)	—	—	—
$D_{AB(2j)}$	0.0131	−0.0131	—	—	−0.0033	0.0033	—	—	−0.0165	0.0165	—	—
	(0.05)	(−0.05)	—	—	(−0.10)	(0.10)	—	—	(−0.55)	(0.55)	—	—
$D_{AB(2jz3)}$	—	—	0.0754*	−0.0754*	—	—	—	0.0000	—	—	—	0.0000
	—	—	(0.60)	(−0.60)	—	—	—	(0.00)	—	—	—	(0.00)
$D_{AB(2jq7)}$	—	0.0000	—	—	—	—	0.0000	—	—	—	0.0000	—
	—	(0.00)	—	—	—	—	(0.00)	—	—	—	(0.00)	—
D_{A2st}	0.0897***	−0.0209	−0.0525***	−0.0163**	0.1083***	−0.0587***	−0.0148*	−0.0348***	0.1326***	−0.0680***	−0.0170*	−0.0476***
	(0.43)	(−0.15)	(−1.00)	(−1.00)	(1.00)	(−1.00)	(−1.00)	(−1.00)	(1.00)	(−1.00)	(−1.00)	(−1.00)
D_{A2j}	0.0090	0.0514***	−0.0461***	−0.0143**	−0.0426***	0.2282***	−0.0554***	−0.1302***	−0.0559***	0.2296***	−0.0457***	−0.1280***
	(0.49)	(0.25)	(−1.00)	(−1.00)	(−0.50)	(1.00)	(−1.00)	(−1.00)	(−0.64)	(1.00)	(−1.00)	(−1.00)
D_{A2jz3}	−0.0823***	−0.0521***	0.1026***	0.0317***	−0.0461***	−0.1189***	−0.0299***	0.1949***	−0.0565***	−0.1190***	−0.0298**	0.2053***
	(−1.00)	(−1.00)	(1.00)	(1.00)	(−1.00)	(−1.00)	(−1.00)	(1.00)	(−1.00)	(−1.00)	(−1.00)	(1.00)
D_{A2jq7}	−0.0165***	0.0216***	−0.0039	−0.0012	−0.0196**	−0.0506***	0.1001***	−0.0299***	−0.0202*	−0.0425***	0.0925***	−0.0298**
	(−1.00)	(1.00)	(−1.00)	(−1.00)	(−1.00)	(−1.00)	(1.00)	(−1.00)	(−1.00)	(−1.00)	(1.00)	(−1.00)
D_{B2st}		0.0025				0.0418***				0.0483***		
		(0.01)				(0.77)				(0.76)		
D_{B2j}		−0.0046				0.0099				−0.0193		
		(−0.03)				(0.05)				(−0.09)		
D_{B2jz3}		−0.0002				−0.0402***				−0.0224		
		(−0.00)				(−0.26)				(−0.13)		
D_{B2jq7}		0.0023				−0.0115				−0.0065		
		(0.14)				(−0.17)				(−0.10)		
D_{AB}	0.0149	−0.147	0.0119	−0.0121*	0.0410***	0.0107	−0.0115	−0.0402***	0.0396**	−0.0107	−0.0065	−0.0224
	(0.06)	(−0.09)	(0.18)	(−0.67)	(0.57)	(0.06)	(−0.17)	(−0.26)	(0.52)	(−0.05)	(−0.10)	(−0.13)

D' values are given in parentheses.
*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

Table 4 Gametic disequilibria (D) between *Est-2* (locus A), *Pept-2*^{a} (locus B) and *Aldox*^{a} (locus C) estimated from 238 gametes derived from wild *Drosophila buzzatii* males collected in Colera (Spain)

	Allele of the *Est-2* locus			
D	*Est-2*^{a}	*Est-2*^{b}*	*Est-2*^{c}	χ^2_1
D_{AB}	0.0558*** (0.505)	−0.0059 (−0.027)	−0.0499*** (−0.426)	19.90*** (d.f. = 2)
D_{AC}	−0.0704*** (−0.626)	0.0356** (0.485)	0.0348*** (0.892)	64.46*** (d.f. = 2)
D_{BC}		−0.0474*** (−0.585)		18.40*** (d.f. = 1)
D_{ABC}	−0.0271*** (−0.625)	0.0232*** (0.625)	0.0040 (0.000)	46.83*** (d.f. = 2)

Relative disequilibria (D') are given in parentheses.
Est-2^b* stands for alleles *Est-2*^{b} and *Est-2*$^{c+}$ pooled.
*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

sions is virtually at its maximum (see the D' values in Table 3).

Pair-wise disequilibria and three-locus disequilibria parameters for the sample of F_1 adults are given in Table 4. Most D values were highly significant. Those between *Est-2* and *Pept-2* were, as expected, very similar to the disequilibria found by analysing a sample of F_1 larvae. In addition, *Aldox* showed strong disequilibria with both *Est-2* and *Pept-2* because of a deficiency of *Aldox*^{a}/*Est-2*^{a} and *Aldox*^{a}/*Pept-2*^{a} gametes.

Discussion

The two populations of *Drosophila buzzatii* studied here, Carboneras and Colera, belong to the primary colonization area of the Old World (Fontdevila *et al.*, 1981). They are at most 300 years old, but could be much more recent, especially Colera, as colonization of the Iberian Peninsula by *D. buzzatii* began from the south (Fontdevila *et al.*, 1981). All the chromosome arrangements and all but one of the allozyme alleles found in Carboneras are also present in Colera. *Est-2*^{d} is relatively rare ($p = 0.03$) in Carboneras and its absence from Colera may be simply a result of the founder effect or drift. The similar polymorphism observed in the two populations is probably a result of gene flow, at the present time or in the recent past, between populations along the Mediterranean coast of the Iberian Peninsula (Fontdevila *et al.*, 1981; Fontdevila, 1989) and selection. The differences in arrangement frequencies observed agree well with the described latitudinal clines in the frequencies of some second chromosome arrangements. The frequency of 2st correlates negatively with latitude ($r = -0.52$, $P < 0.05$) in a set of Old World and New World populations (Ruiz, 1982), and in Australia ($r = -0.42$, $P < 0.10$) (Knibb & Barker, 1988). The Australian populations also show a positive and significant correlation with latitude for 2jz^3 ($r = 0.56$, $P < 0.05$). Furthermore, adults with the 2st or 4st arrangements have, on average, a smaller body size than those carrying inversions (Ruiz *et al.*, 1991; Hasson *et al.*, 1992; Betrán, 1992). Because in *Drosophila* body size usually increases with latitude (Prevosti, 1955; David & Bocquet, 1975) the observed differences in arrangement frequencies between Colera and Carboneras fit well with the latitudinal clines and the effect of inversions on body size.

The marked differences between Carboneras and Colera in allele frequencies may be explained in part by the association with the arrangements. *Est-2*^{a} has a much lower frequency in Colera than in Carboneras. This difference results from the association between *Est-2*^{a} and the 2st arrangement, but not entirely. The frequency of *Est-2*^{a} is greater in Colera than in Carboneras *within* arrangement 2st (100 per cent vs. 72.3 per cent; $\chi^2_1 = 24.5$, $P < 0.001$) whereas it is smaller *within* arrangement 2j (8.1 per cent vs. 53.8 per cent; $\chi^2_1 = 97.48$, $P < 0.001$). These changes may be more parsimoniously attributed to drift or founder effect because a different pattern (*Est-2*^{a} decreasing with latitude within 2st) has been observed by Knibb & Barker (1988) in Australia. *Pept-2*^{a} has a higher

frequency in Colera than in Carboneras. Changes in arrangement frequencies do not explain this difference because *Pept-2*^{a} is associated with 2st. A detailed analysis for *Aldox* is not possible because no direct estimates of the association between *Aldox* and the second chromosome inversions were obtained. *Adh-1*^{b} also presents a higher frequency in Colera than in Carboneras independent of inversions that fits quite well the spatial pattern described for this allele in Australia (Sokal *et al.*, 1987). In addition, *Adh-1* is the only locus which showed a significant difference between the sexes, in agreement also with previous observations in Australia (Barker, 1981; Barker *et al.*, 1986), supporting the idea that variation at *Adh-1* has an adaptive value in *D. buzzatii*.

A different pattern of departures from Hardy–Weinberg is found in the two populations. The excess of heterozygotes shown by the sample of Carboneras was also found in 1987 and 1989 (Quezada-Díaz *et al.*, 1992; Quezada-Díaz, 1993) for some loci located on the second chromosome. Santos (1994) has suggested that multiple niche selection acting on the inversion polymorphism in viability could account for this excess. The general deficiency of heterozygotes observed in Colera (significant for *Est-2* and *Adh-1*) can be explained by a number of factors: null alleles, Wahlund effect, diversifying selection and inbreeding, among others (Gaffney *et al.*, 1990; Santos, 1994). Deficiencies of heterozygous have been repeatedly observed in the Australian populations of *D. buzzatii* (Barker *et al.*, 1986) and interpreted as inbreeding combined, for some loci, with selection. As inbreeding affects all loci equally, heterozygote deficiencies from inbreeding should be the same for all loci and alleles. This hypothesis of homogeneity has been tested by comparing deficiencies among loci treating inbreeding coefficients as correlation coefficients (Crow & Kimura, 1970) and testing them accordingly (Sokal & Rohlf, 1981). Inbreeding coefficients were not homogeneous among loci (males: $F_{Adh\text{-}1} = 0.17$; $F_{Pept\text{-}2} = -0.10$; $F_{Est\text{-}2} = 0.12$; females: $F_{Adh\text{-}1} = 0.15$; $F_{Pept\text{-}2} = 0.04$; $F_{Est\text{-}2} = 0.09$; with $\chi^2_5 = 14.57$, $P < 0.05$). In addition, heterogeneity of inbreeding coefficients was tested within the multiallelic *Est-2* locus. This was performed by means of a goodness of fit test of the observed genotype frequencies to those expected fitting a single inbreeding coefficient (Gaffney *et al.*, 1990). This test gave significant results in females ($F = 0.094$; $\chi^2_2 = 7.58$, $P < 0.05$) but not in males ($F = 0.117$; $\chi^2_2 = 4.84$, $P > 0.05$). These results show that inbreeding cannot be the sole explanation of our observations and that selection is likely to play a role or even be the sole cause of the observed deficiencies (Santos, 1994).

Significant gametic associations between *Est-2* alleles and second chromosome inversions were found in both populations. The association is nearly complete because each chromosome arrangement harbours only one or two *Est-2* alleles. The most parsimonious explanation for these locus–inversion associations is a historical origin coupled with the lack of recombination in the heterokaryotypes (Ishii & Charlesworth, 1977; Nei & Li, 1980) although they are consistent also with a selective role of the *Est-2* variation (Charlesworth, 1974). Nevertheless, the fact that the associations found in Colera and Carboneras are different from those described in the Australian populations (Knibb *et al.*, 1987) suggests an important role of founder events during colonization. Founder effect or drift probably also explain the stronger association *Est-2*–inversion observed in Colera. It would be very interesting to have information about the gametic disequilibria in the original populations of Argentina and Bolivia.

Pept-2 shows a significant linkage disequilibrium with the second chromosome inversions only in Colera. Effective recombination frequencies (taking into account the lack of recombination in *Drosophila* males) between *Pept-2* and the inversion breakpoints are relatively high: 4.2 per cent (with 2j and 2z^3) and 18.15 per cent (with 2q^7). With this amount of recombination, these associations should not be expected unless this locus were involved in epistatic fitness effects (Nei & Li, 1980) or the population suffers strong bottlenecks. The association found in Colera could be generated by random chance if this population were repopulated every year or suffers bottlenecks, in the same way as if the population had a small effective size all the year (Montchamp-Moreau & Katz, 1986). An alternative explanation for the observed *Pept-2*–inversions associations observed in Colera could be that higher levels of linkage disequilibrium from overdominance are theoretically expected in small populations in comparison to large ones (Slatkin, 1977; Yamazaki, 1977; Yamaguchi *et al.*, 1980). At present, no firm conclusions can be reached on the origin of the *Pept-2* inversion disequilibria.

A significant association between *Est-2* and *Pept-2* was found in both populations but from different causes. In Carboneras, the negative association between *Est-2*^{d} and *Pept-2*^{a} results exclusively from the *within* chromosome arrangements component: these two loci are associated within the 2jz^3 arrangement. The effective recombination fraction for the disequilibrium within a given chromosome arrangement equals $u_i c_i$, where u_i is the frequency of the arrangement and c_i is the recombination fraction in the corresponding homokaryotypes (A. Navarro *et al.*, unpublished data).

In the $2jz^3$ arrangement, *Est-2* comes closer to *Pept-2*. A preliminary estimate of 26 per cent ($N = 46$) for the recombination rate between *Est-2* and *Pept-2* in the $2jz^3/jz^3$ homokaryotypes has been obtained (J. E. Quezada-Díaz, personal communication). Taking into account the fact that *Drosophila* males do not recombine, we can estimate the effective recombination frequency for the disequilibrium within $2jz^3$ as 2 per cent. This value is small enough to generate this disequilibrium by drift, especially if we consider that the effective population size for arrangement $2jz^3$ will be six times lower than that of the population as a whole. At Colera, on the other hand, the association between *Est-2* and *Pept-2* results exclusively from the *between* chromosome arrangements component, i.e. it is a simple consequence of the associations between each of these two loci and the second chromosome inversions discussed above.

For *Est-2, Pept-2* and *Aldox*, all pair-wise disequilibria were significant. The disequilibrium was stronger between *Aldox* and *Est-2* than between *Pept-2* and *Est-2*. This is expected from the position of the three loci relative to the inversions (Fig. 1). *Aldox* and *Est-2* are located within the system of inversions while *Pept-2* lies outside all paracentric inversions. As *Est-2* is highly associated with inversions, we can predict the associations of *Aldox* with inversions. $Aldox^b$ is associated with $Est\text{-}2^a$ and, as a consequence, must be associated with 2st; meanwhile $Aldox^a$ is over-represented in 2j, $2jz^3$ and $2jq^7$. A study of the composite genotypic disequilibrium between *Est-2* and *Aldox* in Carboneras (Quezada-Díaz, 1993) yielded a correlation coefficient between $Est\text{-}2^a$ and $Aldox^a$ of -0.0549 ($P < 0.05$). The direction of the association is thus the same in both populations.

The two populations analysed here are the first in which the gametic associations between *Est-2, Pept-2* and *Aldox* in *D. buzzatii* have been described. It is clear that more information from other populations, original and colonized, is needed in order to draw a more complete picture of their associations with the second chromosome inversions.

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