THE GENETICS OF *DROSOPHILA SUBOBSCURA* POPULATIONS. IX. STUDIES ON LINKAGE DISEQUILIBRIUM IN FOUR NATURAL POPULATIONS

M. LOUKAS, C. B. KRIMBAS AND Y. VERGINI

Department of Genetics, Agricultural College of Athens, Greece

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ABSTRACT

Gametic frequencies were obtained in four natural populations of *D. subobscura* by extracting wild chromosomes and subsequently analyzing them for inversions and allozymes. The genes *Lap* and *Pept-1*, both located within the same inversions of chromosome O, were found in striking nonrandom associations with them of the same kind and degree in all populations studied. On the contrary, the gene *Acph*, also located within the previously mentioned inversions, was found in linkage disequilibrium with them only in two populations and of opposite directions. This is also the case for the genes *Est-9* and *Hk*, both located within chromosome *E* inversions. While the gene *Est-9* was in strong linkage disequilibrium with the inversions, of the same kind and degree in all populations studied, *Hk* was found to be in linkage equilibrium.

Allele frequencies for the 29 genes studied do not show geographical variation except for the genes *Lap*, *Pept-1* and *Est-9*, the ones found in linkage disequilibria with the geographically varying gene arrangements. Although mechanical or historical explanations for these equilibria cannot be ruled out, these data cannot be explained satisfactorily by the "middle gene explanation," which states that loci displaying such linkage disequilibria are the ones located near the break points of inversions, while the ones displaying linkage equilibria with them are located in the middle of them.

There is no evidence for consistent linkage disequilibria between pairs of loci, except for the closely linked genes of the complex locus, *Est-9*. This would imply, if it is not a peculiarity of the *Est-9* complex, that the linkage disequilibria are found only between very closely linked loci or that, for less closely linked genes, the associations are too weak to be detected by the usual samples sizes.

THE possibility of complications because of linkage disequilibria is the major difficulty encountered in planning experiments and interpreting results in order to decide between the two alternative theories of population structure, the classical and the selectionist (or balanced). Most, if not all, of the available methods (including the one testing the heterogeneity of the *F* values) do not permit an unambiguous decision as to whether a gene studied is indeed selected, since it is possible that its behavior could be due to another gene in linkage disequilibrium with it. Not only selection, but also drift and embedding effects can produce and reinforce such disequilibria, at least temporarily, even with entirely "selectively neutral" genes.

Furthermore, arguments on the amount of the genetic load (not absolutely convincing now) have pushed many workers, in order to save the selectionist theory, to propose truncation and other models of selection that generate important epistatic interactions (SVED, REED and BODMER 1967; KING 1967; MILKMAN 1967; WILLS 1978). These interactions are expected to produce important linkage disequilibria, at least between the closely linked genes that are selected. Finally, the FRANKLIN and LEWONTIN (1970) model of multiplicative effects between numerous genes predicts a highly organized chromosome structure based on linkage disequilibria of closely or even remotely linked genes. This model switches our interest from the gene to the chromosome as the fundamental unit of selection.

It seems, thus, highly desirable to continue and extend the studies of natural populations to decide, first, whether such disequilibria do in fact exist. The nonexistence of such disequilibria could greatly simplify experimental approaches to the study of the genetic structure of natural populations, because it would prove that the difficulties considered above are imaginary. On the other hand, if such disequilibria do exist, it would be easier to study population structure by taking advantage of them, that is to say, by investigating the causes for their existence: selection, sample error, drift or historical accident. In order to be able to answer all these questions, we report here a large-scale simultaneous study of the inversion structure of the chromosomes and their allelic content. Four natural populations of *Drosophila subobscura* have been studied for 29 enzyme loci and the inversion structure of the five chromosomes (with one exception) in such a way that, for every gene sampled, we knew its electrophoretic allele, the electrophoretic alleles of the other genes studied on that same chromosome, and the gene arrangement of that chromosome.

MATERIALS AND METHODS

The populations: The following four natural populations of *D. subobscura* have been sampled in August and September 1975, using banana traps: *Sussex*, England in a woodland of Quercus and Ulmus near the University; *Barcelona*, Spain, in a *Pinus* woodland 10 km from the city; *Mount Parnes*, 40 km from Athens, Greece, in a fir forest (*Abies cephallonica*); and *Preveza*, 5 km from the town in an orange orchard.

The genes, their location and electrophoretic techniques: The polymorphism of 29 enzyme loci was studied: *Lap*, *Pept-1*, *Acph*, *Xdh*, *ME*, *Ao*, *Odh*, *Est-5* (all on chromosome *O*); *Est-3*, *Est-7*, *Aph*, *Pgm*, *Idh*, Diaphorase-1 (all on chromosome *J*); *Mdh*, *Adh*, *αGPD* (all on chromosome *U*); *Hk-1*, *Pept-3*, *Phi* and the complex gene *Est-9* (all on chromosome *E*); *6-PGD*, *G-6-PD* (both on the sex chromosome *A*); and *Ald*, *Fum*, *ATPase*, *Hk-3*, Diaphorase-2, and *To* (all unlocated). The electrophoretic techniques for detecting the enzymes and complete linkage data are given in LOUKAS and KRIMBAS (1979) and in LOUKAS *et al.* (1979), which completes and corrects the older data of LOUKAS (1974).

The crosses: To study the polymorphism of chromosomes *O*, *J*, *U* and *A* and that of the genes located on them, we crossed separately every wild-caught male to virgin females from a stock homozygous for the recessive mutants cherry eyes (*ch*) and curled wings (*cu*), both on chromosome *O*. This *ch cu* stock is homozygous for gene arrangements in all five chromosomes (O_{3+4}, J_{ST}, U_{ST}, E_{ST}, A_{ST}) and also homozygous for all the loci analyzed in this study. The examination of salivary gland chromosomes of the F_1 permitted us to establish the chromosome arrangement of the sex chromosome *A* of the original wild male, since his daughters had one

such chromosome from him and the other from the *ch cu* stock. These F_1 females were also assayed for the two sex linked genes. One F_1 male from this cross was recrossed to *ch cu* females and the salivary glands of one to eight early pupae were examined from each such cross in order to assay the gene arrangement of the chromosomes *O*, *J* and *U* isolated from the original wild male. If no heterozygote for inversions at a chromosome was detected among all eight preparations, the wild chromosome was assigned the gene arrangement of the *ch cu* strain. The same procedure was followed in detecting allozyme variation at loci other than those located on chromosome *O* and detected in the adult stage. One to eight progeny had to be electrophoresed in order to obtain the allelic form carried by the wild chromosome. For loci detectable at the adult stage and located on chromosome *O*, it was enough to run one "wild-type" offspring of the back cross.

The procedure was more complicated for detecting variation of the *E* chromosome because of the difficulties in assigning allozyme types of the *Est-9* gene complex. We used the strain *p sj pl* (poppy eyes, six-jointed tarsi and plexus wings), which is homozygous for the Standard gene arrangement of the *E* chromosome (E_{ST}), the *35* type of *Est-9*, and also for the three other genes located on chromosome *E*. Every wild-caught male was crossed separately to virgin *p sj pl* females, and a single male from each cross was backcrossed to the *p sj pl* strain. From the progeny of this cross, one to eight early pupae were analyzed by salivary gland preparations, in order to ascertain the structural type of the isolated wild *E* chromosome. The wild-type adults were electrophoresed for all genes located in chromosome *E*. Thus, it was possible to define the *Est-9* type, except for the *3* and *5* "alleles." In order to define completely the *Est-9* type, one wild-type son of this last cross was crossed to virgin females of a strain homozygous for the type *47* of *Est-9*. Eight adults from the progeny were electrophoresed. Half of these flies were expected to carry the wild chromosome. This laborious procedure permitted the complete identification of the *Est-9* type.

RESULTS AND ANALYSES

Chromosomal variation: Table 1 shows the frequencies of the different gene arrangements found in the four populations studied. Only two comments will be made: first, the different populations are differentiated, not only in frequencies, but also in the kind of gene arrangements found. This finding is not new, since many workers have noted north-south clines of frequencies in gene arrangements in all chromosomes. This will be relevant, however, in explaining the gene frequency data mentioned below. The second remark concerns the linkage disequilibria observed between nonoverlapping inversions: these disequilibria were found for inversions A_1 and A_2 of the sex chromosome *A* and also between inversions belonging to the two segments of chromosome *O*. In the first segment (map sections 90–99 of the KUNZE-MÜHL and MÜLLER 1958 map) the gene arrangements found are O_{3+4}, O_{ST}, O_{3+4+8} and O_{3+4+16}, while in the second (map sections 77–90) they are O_1, O_2, O_{22}, O_7, etc. A continuous line below the numbers shows the overlapping inversions. Thus A_1 and A_2 in our material are never found together, and O_1 is always associated with O_{3+4} in the O_{3+4+1} gene arrangement. The same is true for O_2, O_{22}, O_7, etc. We know that in spite of the distance separating them, a nearly absolute linkage in the heterozygote females for two nonoverlapping inversions is responsible for these disequilibria (KRIMBAS and ZOUROS 1969; SPERLICH and FEUERBACH 1969; SPERLICH and FEUERBACH-MRAVLAG 1974).

Genic variation. Of the 29 genes studied, five (17%) displayed no electrophoretic variation in any population: *Fum*, ATPase. *Hk-3*, Diaphorase-2 and *To*.

TABLE 1

Gene arrangement frequencies in chromosomes O, J, U, E *(autosomes) and* A *(sex chromosome)*

			Sussex	Mt. Parnes	Preveza	Barcelona
O_{ST}	(I_{ST}	$+II_{ST}$)*	0.440	0.126	0.188	0.284
O_6	(I_{ST}	$+II_6$)	0	0	0.005	0
O_7	(I_{ST}	$+II_7$)	0	0	0	0.016
O_{3+4}	(I_{3+4}	$+II_{ST}$)	0.425	0.526	0.455	0.310
O_{3+4+1}	(I_{3+4}	$+II_1$)	0	0.137	0.168	0.037
O_{3+4+2}	(I_{3+4}	$+II_2$)	0	0.042	0.030	0.005
O_{3+4+22}	(I_{3+4}	$+II_{22}$)	0	0.111	0.109	0.021
O_{3+4+6}	(I_{3+4}	$+II_6$)	0.010	0	0	0
O_{3+4+7}	(I_{3+4}	$+II_7$)	0.005	0	0.020	0.182
O_{3+4+8}	(I_{3+4+8}	$+II_{ST}$)	0.120	0.047	0.020	0.139
$O_{3+4+16+2}$	(I_{3+4+16}	$+II_2$)	0	0.011	0.005	0.005
Sample size			200	190	202	187
J_{ST}			0.375	0.126	0.173	0.246
J_1			0.625	0.869	0.817	0.754
J_{3+4}			0	0.005	0.010	0
Sample size			200	190	202	187
U_{ST}			0.055	0.021	0.050	0.059
U_1			0.030	0	0.005	0.011
U_2			0	0.026	0	0
U_{1+2}			0.875	0.406	0.346	0.492
U_{1+2+3}			0	0.011	0.015	0
U_{1+2+6}			0.005	0.384	0.495	0.032
U_{1+2+7}			0	0.084	0.025	0
U_{1+2+8}			0.035	0.068	0.064	0.406
Sample size			200	190	202	187
E_{ST}			0.610	0.113	0.224	0.236
E_8			0.005	0.236	0.224	0.070
E_{1+2}			0.360	0.072	0.054	0.194
E_{1+2+9}			0	0.518	0.478	0.220
$E_{1+2+9+3}$			0	0	0	0.027
$E_{1+2+9+12}$			0.025	0.061	0.020	0.253
Sample size			200	195	205	186
A_{ST}				0.500	0.460	0.480
A_1				0.190	0.360	0.160
A_2				0.310	0.180	0.360
Sample size				100	100	100

* The parentheses give the gene arrangement type of the first and the second segment of chromosome *O*.

Another nine genes (32%) did display electrophoretic variation; however, in all populations studied, their commonest allele had a frequency equal or higher than 0.95. These genes are *Odh*, *Idh*, Diaphorase-1, *Mdh*, *Adh*, *aGPD*, *Pept-3*, *Phi* and *Ald*. Thus, by the usual criteria, 49% of the genes studied can be considered monomorphic. A group of three genes (10%) lies in the middle of the monomorphic and polymorphic categories, since in some populations these genes behave as monomorphic, but in others as polymorphic. Generally speaking the frequency of their commonest allele is around 0.95. These genes are: *Est-7*, *6-PGD* and *G6PD*. Finally, there is the group of 12 genes (41%) that are polymorphic in all populations studied: *Est-9*, *Lap*, *Pept-1*, *Acph*, *Xdh*, *ME*, *Ao*, *Est-5*, *Est-3*, *Aph*, *Pgm* and *Hk-1*. Although the percentage of polymorphic genes found in *D. subobscura* seems to be in the higher part of the range, it is generally in good agreement with the findings of other workers on different Drosophila species.

In Table 2, we give the allelic frequencies of all the genes that are not completely monomorphic, except for *Est-9*, whose frequencies are given in Table 3 in a different way. An inspection of these two tables and the tests performed shows that all populations are alike in allelic frequencies for all genes studied, except for the following: *Lap*, *Pept-1*, *Acph* and *Est-9*. In performing these tests, we pooled together alleles starting with those having the lowest frequency and proceeding until the expectation at every cell was higher than 5. With the exception of these four genes, the complete homogeneity found reminds us of similar results in *D. pseudoobscura*.

The Sussex population, which is nearer the northern margin of the geographical distribution than the other three populations, differs neither in the kind of predominant alleles nor in their frequencies! The same is true for the Crete population near the southern margin (unpublished data). These observations are in direct contradiction with CARSON's hypothesis of homoselection first, because a monomorphism or a decline of genetic variability is expected in the marginal populations and second, because a different kind of monomorphism is expected in the northern and in the southern range of the geographical distribution of the species, if climate plays any selective role. Thus, except for the four above-mentioned genes, the results are not in agreement with CARSON's hypothesis. We will explain below why these four genes constitute such an exception.

Associations between genes located on the same chromosome: The detailed data of this study are too bulky to be presented here. They are deposited at the Department of Genetics of the Agricultural College of Athens. Also, the results of 163 tests concerning departure from linkage equilibrium between alleles of two genes located on the same chromosome are not presented here, as requested by the editors. Each of these tests concerns two genes (in most of these tests, alleles have been grouped in two classes) in one population. In some instances where the expected number of one class was less than five, we did perform the test, following LEWONTIN and FELSENSTEIN (1965), who demonstrated that even in these cases the test is not greatly affected. However, in all 2×2 tables, when

TABLE 2

Allelic frequencies at twenty-three loci displaying more than one allele

	Sussex	Mt. Parnes	Preveza	Barcelona
Lap, chr. *O*				
1.25	0.010	0	0.015	0.005
1.18	0.060	0	0.020	0.027
1.11	0.060	0.069	0.084	0.107
1.06	0.110	0.063	0.114	0.118
1.00	0.715	0.847	0.727	0.690
0.92	0	0.005	0.005	0
0.86	0.045	0.016	0.025	0.048
0.69	0	0	0.010	0.005
**Pept-1*, chr. *O*				
1.60	0.035	0.053	0.015	0.011
1.00	0.455	0.253	0.307	0.347
0.40	0.510	0.694	0.678	0.642
**Acph*, chr. *O*				
2.00	0.010	0	0.005	0.005
1.88	0.040	0.047	0.044	0.043
1.00	0.855	0.853	0.797	0.866
0.54	0.085	0.100	0.144	0.064
0.25	0.010	0	0.010	0.022
**Mdh*, chr. *O*				
1.08	0	0.005	0.010	0.016
1.06	0.015	0.016	0.030	0.016
1.04	0.130	0.142	0.134	0.085
1.00	0.685	0.674	0.653	0.658
0.96	0.100	0.116	0.124	0.102
0.92	0.060	0.037	0.039	0.086
0.86	0.010	0.010	0.010	0.037
**ME*, chr. *O*				
1.10	0.025	0.016	0.025	0.011
1.00	0.905	0.947	0.906	0.930
0.94	0.055	0.037	0.064	0.054
0.75	0.010	0	0.005	0.005
0.65	0.005	0	0	0
**AO*, chr. *O*				
1.10	0	0.016	0.029	0.048
1.08	0.080	0.100	0.050	0.075
1.05	0.225	0.237	0.198	0.139
1.00	0.555	0.547	0.569	0.567
0.95	0.100	0.053	0.089	0.112
0.91	0.035	0.037	0.050	0.037
0.82	0.005	0.010	0.015	0.022
**Odh*, chr. *O*				
1.14	0.040	0.016	0.025	0.022
1.00	0.950	0.979	0.965	0.973
0.86	0.005	0.005	0.010	0.005
0.58	0.005	0	0	0

TABLE 2—Continued

	Sussex	Mt. Parnes	Preveza	Barcelona
Est-5, chr. *O*				
1.25	0.030	0	0.005	0
1.06	0.045	0.026	0.020	0.005
1.00	0.355	0.405	0.470	0.390
0.90	0.495	0.527	0.450	0.519
0.86	0.075	0.021	0.045	0.069
0.78	0	0.021	0.010	0.016
Sample size for every gene located on chromosome *O*:	200	190	202	187
Est-3, chr. *J*				
1.30	0.010	0	0	0
1.20	0.305	0.289	0.257	0.235
1.00	0.650	0.674	0.728	0.711
0.90	0.035	0.037	0.015	0.048
0.70	0	0	0	0.005
**Est-7*, chr. *J*				
1.04	0.050	0.058	0.050	0.043
1.02	0	0.010	0.010	0.005
1.00	0.950	0.932	0.940	0.952
**Aph*, chr. *J*				
1.27	0	0	0.005	0.005
1.00	0.555	0.453	0.465	0.519
0.73	0.440	0.547	0.530	0.476
0.46	0.005	0	0	0
Pgm, chr. *J*				
1.58	0	0	0	0.011
1.32	0.040	0.037	0.064	0.048
1.10	0.005	0.005	0	0
1.00	0.930	0.921	0.926	0.920
0.90	0	0	0.005	0
0.71	0.025	0.037	0.005	0.016
0.42	0	0	0	0.005
**Idh*, chr. *J*				
1.17	0.030	0.011	0	0.011
1.00	0.970	0.984	0.995	0.989
0.83	0	0.005	0.005	0
**Diaph-1*, chr. *J*				
1.00	1.000	1.000	1.0000	0.984
0.85	0	0	0	0.016
Sample size for every gene located on chromosome *J*:	200	190	202	187
**Mdh*, chr. *U*				
1.35	0.005	0	0.015	0.027
1.00	0.995	0.995	0.985	0.973
0.65	0	0.005	0	0
**Adh*, chr. *U*				
1.67	0.035	0.005	0	0.021
1.00	0.965	0.995	1.000	0.979

TABLE 2—Continued

	Sussex	Mt. Parnes	Preveza	Barcelona
**αGPDH*, chr. *U*				
1.38	0.010	0.011	0.010	0.005
1.00	0.990	0.989	0.990	0.990
0.60	0	0	0	0.005
Sample size for every gene located on chromosome *U*:	200	190	202	187
Hk-1, chr. *E*				
1.43	0.010	0.005	0	0
1.27	0.425	0.400	0.341	0.419
1.00	0.430	0.492	0.542	0.484
0.73	0.135	0.103	0.117	0.097
**Pept-3*, chr. *E*				
1.08	0	0.005	0	0
1.00	0.985	0.990	0.995	0.989
0.92	0.005	0	0	0.005
0.83	0.010	0.005	0.005	0.005
**Phi*, chr. *E*				
1.80	0	0	0	0.005
1.00	1.000	1.000	1.000	0.995
Sample size for every gene located on chromosome *E*:	200	195	205	186
**6-PGD*, chr. *A*				
1.56		0.010	0.010	0
1.27		0	0	0.010
1.00		0.960	0.930	0.910
0.78		0.030	0.060	0.070
0.33		0	0	0.010
**G-6-PD*, chr. *A*				
1.17		0	0.030	0
1.07		0	0.040	0.020
1.00		1.000	0.930	0.980
Sample size for every gene located on chromosome *A*:		100	100	100
**Ald*, autos.				
1.00	1.000	1.000	1.000	0.995
0.74	0	0	0	0.005
Sample size:	200	190	202	187

* Genes forming hybrid enzymes in heterozygotes are marked with asterisks.

one class was less than five, we did not use the χ^2 test, but calculated Fisher's exact probability by a quick and extremely accurate method invented by JOHN SOURDIS of our Department. This method permits the direct calculation of probabilities without resorting to logarithmic tables of factorials. It is based on two observations: first, that a relatively small number of configurations of 2×2 contingency tables exists in most of the cases (the method provides an easy way to enumerate all of them), and second, that the ratios of the probabilities of two

TABLE 3

The frequencies of alleles of the Est-9 *"complex gene" in different gene arrangements (overall frequencies of these alleles in every population can be computed from the data presented in this Table)*

Populations	E_S	E_{ST}	Chromosomal arrangement E_{1+2}	E_{1+2+9}	$E_{1+2+9+12}$	$E_{1+2+9+3}$
Sussex	*36* 1.000	*35* 0.279	*4* 0.305		*25* 0.600	
		4 0.254	*34* 0.138		*24* 0.200	
		34 0.123	*35* 0.138		*36* 0.200	
$N = 200$		*5* 0.074	*5* 0.083			
		3 0.058	*36'* 0.056			
		46 0.041	*46* 0.042			
		36 0.025	*3* 0.028			
		45 0.025	*36* 0.028			
		4' 0.025	*45* 0.028			
		37 0.016	*4'5* 0.028			
		38 0.016	*1'4* 0.014			
		1' 0.008	*24* 0.014			
		1'4 0.008	*34'* 0.014			
		25 0.008	*37* 0.014			
		27 0.008	*44'* 0.014			
		36' 0.008	*47* 0.014			
		47 0.008	*47* 0.014			
		49 0.008	*49* 0.014			
		4'5 0.008	*4'* 0.014			
	$N = 1$	$N = 122$	$N = 72$	$N = 0$	$N = 5$	$N = 0$
Mt. Parnes	*34* 0.369	*34* 0.227	*34* 0.286	*24* 0.317	*25* 0.834	
	36 0.261	*35* 0.227	*35* 0.215	*1'4* 0.168	*1'4* 0.083	
	4 0.152	*24* 0.092	*36* 0.143	*25* 0.128	*26* 0.083	
$N = 195$	*35* 0.065	*26* 0.092	*46* 0.143	*34* 0.109		
	4'5 0.043	*47* 0.092	*26* 0.071	*35* 0.109		
	3 0.022	*3* 0.045	*4* 0.071	*1'5* 0.059		
	347 0.022	*36'* 0.045	*47* 0.071	*14* 0.020		
	37 0.022	*38* 0.045		*4* 0.020		
	46' 0.022	*4* 0.045		*1'3* 0.010		
	5 0.022	*46* 0.045		*1'7* 0.010		
		5 0.045		*26* 0.010		
				27 0.010		
				2'4 0.010		
				3 0.010		
				36 0.010		
	$N = 46$	$N = 22$	$N = 14$	$N = 101$	$N = 12$	$N = 0$
Preveza	*34* 0.500	*34* 0.239	*34* 0.364	*24* 0.357	*25* 0.750	
	4 0.239	*4* 0.195	*4* 0.364	*1'4* 0.153	*5* 0.250	
	36 0.174	*35* 0.174	*24* 0.091	*25* 0.153		
$N = 205$	*35* 0.043	*46* 0.087	*3* 0.091	*1'5* 0.102		
	24 0.022	*3* 0.065	*45* 0.091	*34* 0.072		

TABLE 3—Continued

Populations	Chromosomal arrangement E_8	E_{ST}	E_{1+2}	E_{1+2+9}	$E_{1+2+9+12}$	$E_{1+2+9+8}$
	26' 0.022	*5* 0.043		*35* 0.041		
		24 0.022		*4* 0.031		
		29 0.022		*26* 0.021		
		2'5 0.022		*15* 0.010		
		36 0.022		*1'47* 0.010		
		36' 0.022		*14* 0.010		
		37 0.022		*34'* 0.010		
		38 0.022		*36* 0.010		
		39 0.022		*46* 0.010		
		47 0.022		*4'5* 0.010		
	$N = 46$	$N = 46$	$N = 11$	$N = 98$	$N = 4$	$N = 0$
Barcelona	*34* 0.384	*4* 0.227	*4* 0.306	*25* 0.244	*25* 0.617	*34* 0.800
	36 0.308	*35* 0.182	*34* 0.111	*1'4* 0.219	*4* 0.085	*35* 0.200
	4 0.308	*34* 0.136	*35* 0.083	*24* 0.171	*1'5* 0.043	
$N = 186$		*46* 0.091	*48* 0.083	*35* 0.122	*24* 0.043	
		45 0.068	*5* 0.083	*34* 0.098	*35* 0.043	
		4' 0.068	*3* 0.055	*1'5* 0.073	*26* 0.021	
		37 0.045	*47* 0.055	*1'8* 0.024	*2'7* 0.021	
		47 0.045	*4'* 0.055	*27* 0.024	*34'* 0.021	
		5 0.045	*24* 0.028	*47* 0.024	*36* 0.021	
		3 0.023	*34'* 0.028		*45* 0.021	
		36 0.023	*36* 0.028		*46* 0.021	
		57 0.023	*37* 0.028		*4'* 0.021	
		6' 0.023	*45* 0.028		*5* 0.021	
	$N = 13$	$N = 44$	$N = 36$	$N = 41$	$N = 47$	$N = 5$

"successive" configurations have a simple form. The sum of the relative probabilities for each configuration (calculated from these ratios) permits the estimation of the exact probability for each configuration. Every probability calculated by this method is shown by an asterisk to its left.

These 163 tests involve 120 tests with pairs of genes located on chromosome *O* (in 98 of these tests, all gene arrangements of chromosome *O* were included in the sample; in 10 of them, the association of the genes was tested only within the O_{ST} gene arrangement; in 12 only within O_{3+4}); 28 tests of pairs located on chromosome *J*; and 15 tests of pairs located on chromosome *E* (in four of these, all chromosome *E* gene arrangements were considered; in three only E_{ST}; in two only E_8; in two only E_{1+2}; in three only E_{1+2+9} and in one only $E_{1+2+9+12}$). Genes located on chromosomes *A* and *U* have not been tested, since at least one of them in each pair shows an extremely low degree of polymorphism. No systematic linkage disequilibria were detected in these tests. Only six of them were found statistically significant at the 0.05 level: (a) Between *Xdh* and *Ao* in the Barcelona population ($P < 0.01$). The associations between *Xdh* and *Ao* alleles described by ZOUROS and KRIMBAS (1973) were not found in our study, although care was taken to cross-check with the previous authors the identity of these

genes and alleles. These correspondences are the following (question marks indicate dubious correspondences):

Xdh		*Ao*	
Present study	ZOUROS and KRIMBAS	Present study	ZOUROS and KRIMBAS
1.08		1.10	
	0.96?		0.91?
1.06		1.08	
1.04	0.94	1.05	0.89
1.00	0.92	1.00	0.87
0.96	0.90	0.95	0.85
0.92	0.89?	0.91	0.83?
0.86	0.88?	0.82	0.82?

This is not only true for the four populations reported here, including Mt. Parnes, but also for Crete (unpublished observations): these latter two were the only ones studied by ZOUROS and KRIMBAS. Thus, the earlier data are not confirmed; however; there is still a tendency to observe more than the expected associations of fast alleles of both these genes, and the same is true for the slow ones, exactly as in the previous study, but in a statistically nonsignificant way. The disequilibrium in the Barcelona population does not seem to conform to these observations. It disappears if we exclude from the data the O_{3+4+7} gene arrangement (inversion O_7 seems to contain *Xdh* and *Ao*): $\chi^2 = 8.119$ for 4 d.f., $0.05 < P < 0.10$. Within O_{3+4+7} there is also no disequilibrium. This seems to indicate that O_7 mechanically ties up some alleles, creating in this population a disequilibrium between these genes. This disequilibrium is probably due to drift.

(b) Between *Pept-1* and *Lap* in the Sussex population ($P < 0.02$). The Sussex population is polymorphic for gene arrangements at segment I of chromosome *O* where these genes lie: O_{ST} (0.44), O_{3+4} (0.44), and O_{3+4+8} (0.12). It will be shown below that $Pept\text{-}1^{1.00}$ is in strong association with O_{ST} and that $Lap^{1.25}$, $Lap^{1.18}$, $Lap^{1.11}$ and $Lap^{1.06}$ also strong associate with O_{ST}; whereas, $Pept\text{-}1^{0.40}$ and $Lap^{1.00}$ associate strongly with O_{3+4} and O_{3+4+8}. If we examine the association of these genes within O_{ST} chromosomes or within O_{3+4}, the disequilibrium between these genes disappears completely. Thus, it is not a primary phenomenon, but one due to the disequilibria between genes and inversions. The inversion frequencies in the other populations studied are such that they do not lead to a significant result when the association of these genes is examined.

(c) Between *Lap* and *Acph* within the O_{ST} gene arrangement in the Barcelona population ($P < 0.01$). In two other cases, Preveza and Sussex (Mt. Parnes has too few O_{ST} to be tested), the correlation between the two genes is of the same direction, although not significant. The same is true for three out of four tests for the O_{3+4} gene arrangement, Sussex being in the contrary direction. It is possible that an increased sample would make the situation clearer. As it stands, drift seems the simplest explanation, although selection cannot be excluded.

The three cases mentioned above are significant at the 0.02 or 0.01 probability level. There are three other cases significant at the 0.05 level:

(d) Between *Ao* and *Pept-1* in the Barcelona population ($P < 0.05$),

(e) Between *Est-7* and *Aph* in the Barcelona population ($P = *0.036$),

(f) Between *Aph* and *Pgm* in the Preveza population ($P < 0.05$).

These cases could probably be attributed to sampling errors. We should indeed expect some tests to be significant at the 0.05 level among the 163 ones reported. (It should, however, be noted that these 163 tests are not independent).

It is remarkable that from these tests we cannot make a plausible argument for epistatic selection.

Associations between genes of the Est-9 *gene complex*: *Est-9* is a complex of tightly linked genes. The distance between these genes has been estimated as 0.002 cM. For every *Est-9* gene, according to this interpretation, two alleles are found, an active and a silent one. Population studies revealed that every chromosome *E*, on which *Est-9* is located, bears one to three active alleles of this complex gene, in most cases two. *Est-9* "allele configurations" are in linkage disequilibrium with inversions of the *E* chromosome (LOUKAS and KRIMBAS 1975; LOUKAS *et al.* (1979).

Another interesting aspect is the linkage disequilibrium between the *Est-9* genes. We can test the random occurrence of two zones together either by computing chi-squares or by estimating Fisher's exact probability. Seventy-four such tests are presented in Table 4. Every test was done within one inversion type in order to avoid phenomena of secondary associations due to linkage disequilibria of *Est-9* configurations with inversions. Thirty of the 74 tests are significant at the 0.05 level, 20 at the 0.01 level. Three trends are recognized:

(1) Fast zones (staining slowly) tend not to occur on the same chromosome (zones 1, 2, 3). Seven out of seven such tests are statistically significant.

(2) Slow zones (staining faster) tend not to occur on the same chromosome (4, 5, 6). Thirteen of 23 such tests are statistically significant.

(3) Zone 3 tends not to occur on the same chromosome with zone 4. Six out of the 11 such tests shown in Table 4 are statistically significant. The other five tests, although nonsignificant, were also on the same direction as the significant ones, showing a depletion of 34 configurations. We tried two other tests for not very common gene arrangements (which for that reason are not shown in Table 5): E_{1+2} in Mt. Parnes and E_8 in Barcelona. Both are nonsignificant, but display a depletion of 34 configurations.

Twenty-six out of the 30 significant tests are due to the three trends explained above.

It seems (with the exception of the occurrence of 3 with 4) that the trend is to get a fast and a slow-moving zone on the same chromosome. The same finding has been reported by LOUKAS and KRIMBAS (1975) for the Mt. Parnes, Crete and Finland populations.

In spite of the fact that crossing over between these genes is restricted (and more so because of inversion types), it seems difficult in this cases to avoid selection as an explanation. We do not know how ancient this polymorphism is and whether it has reached a steady state. However, if the polymorphism is younger than the inversions, we cannot explain why the same patterns of associations between *Est-9* "alleles" are found within all the inversions. On the other hand, if it is older than the inversions, it is a puzzle why all inversions display the same patterns of associations, since each one of them should have captured only

TABLE 4

Tests for linkage disequilibrium between Est-9 *alleles computed either by chi-squares (all with one degree of freedom) or by Fisher's exact probability; every test was done within one inversion type of one population*

Sussex E_{ST}
- *3* with *4****
- *3* with *5****
- *3* with *6*
- *4* with *5****
- *4* with *6*
- *5* with *6**

Sussex E_{1+2}
- *3* with *4****
- *3* with *5*
- *3* with *6*
- *4* with *5****
- *4* with *6*
- *5* with *6*

Mt. Parnes E_{S}
- *3* with *4*
- *3* with *5*
- *3* with *6**
- *4* with *5***
- *4* with *6****
- *5* with *6*

Mt. Parnes E_{1+2+9}
- *1'* with *3***
- *1'* with *4*
- *1'* with *5*
- *2* with *3****
- *2* with *4*
- *2* with *5*
- *3* with *4**
- *3* with *5*
- *4* with *5****

Preveza E_{ST}
- *3* with *4**
- *3* with *5*
- *3* with *6*
- *4* with *5****
- *4* with *6*
- *5* with *6*

Preveza E_{S}
- *3* with *4*
- *3* with *6*
- *4* with *6****

Preveza E_{1+2+9}
- *1'* with *3**
- *1'* with *4*
- *1'* with *5*
- *1'* with *6*
- *2* with *3****
- *2* with *4*
- *2* with *5*
- *2* with *6*
- *3* with *4*
- *3* with *5*
- *3* with *6*
- *4* with *5****
- *4* with *6*
- *5* with *6*

Barcelona E_{ST}
- *3* with *4***
- *3* with *5*
- *3* with *6*
- *4* with *5***
- *4* with *6*
- *5* with *6*

Barcelona E_{1+2}
- *3* with *4***
- *3* with *5*
- *4* with *5**

Barcelona E_{1+2+9}
- *1'* with *3**
- *1'* with *4*
- *1'* with *5*
- *2* with *3**
- *2* with *4*
- *2* with *5*
- *3* with *4*
- *3* with *5*
- *4* with *5****

Barcelona $E_{1+2+9+12}$
- *2* with *3***
- *2* with *4**
- *2* with *5***
- *3* with *4*
- *3* with *5*
- *4* with *5**

* Indicates significance at 0.05 probability level, ** at 0.01, and *** at 0.001.

one configuration at its origin, and exchange between them by double crossovers would be rarer than recombinations between *Est-9* genes.

Associations between genes and inversions of the same chromosome: Ninety tests, shown in Table 5, were performed in order to detect departures from random associations between genes and gene arrangements of the same chromosome. In Table 5 for each test are shown: (1) the gene and the chromosome structures tested, in such a way that the alleles and gene arrangements are indicated (the remaining alleles or gene arrangements grouped in one class); (2) the population to which each particular test refers; (3) the value of the chi-square computed; and (4) the degrees of freedom and the probability to get such or a worse result given random association. *Pept-1*, *Lap* and *Acph*, located in segment I of chromosome *O*, have been tested with both *O* chromosome segments, as well as genes *Est-5*, *Ao*, *Xdh*, *ME* and *Odh*, located in segment II of chromosome *O*. Twenty seven of the tests performed are significant at the 0.05 level.

Some of the significant tests are those for a gene-gene arrangement couple in only one of the four populations studied: (a) Thus, *Ao* shows linkage disequilibrium with segment's II inversions of chromosome *O* in the Mt. Parnes population (No. 16). The significance disappears when inversion O_2 is excluded from the test ($\chi^2 = 4.394$, 2 d.f. $0.10 < P < 0.20$). *Ao* is probably located within inversion O_2. In the Mt. Parnes sample, $Ao^{1.00}$ is found more often than expected with the standard gene arrangement, and the other alleles with O_2. (b) *Xdh* shows a similar disequilibrium with the inversions of segment II of chromosome *O* in the Barcelona sample (No. 21). Here again, the significance disappears when inversion O_7 is excluded from the sample (Fisher's exact probability, 0.347). *Xdh* is probably located within this inversion. In the Barcelona sample, $Xdh^{1.00}$ is more often found with the standard gene arrangement, the other alleles with inversion O_7. (c) *6-PGD* shows a linkage disequilibrium with chromosome *A* inversions in the Barcelona sample, which it does not show in the Preveza sample (these two samples are the only ones tested). We do not know the exact location of *6-PGD*. The rare alleles, *1.27*, *0.78* and *0.33*, are more often in A_1 or A_2 gene arrangements than expected. (d) *ME* also shows a linkage disequilibrium with inversions of segment I of chromosome *O* in the Barcelona sample (No. 58, $P = *0.046$). *ME* is not located within these inversions and does not display such a linkage disequilibrium in the other populations sampled. The significance also disappears in the Barcelona sample if the test is performed within a ST gene arrangement in segment II of chromosome *O*. ME is probably located within these inversions, but a direct test with them does not show a significant departure from linkage equilibrium (No. 24, $P = *0.190$).

It is not possible to make a plausible case for selection in the four cases mentioned above. Drift is a possible alternative.

In four other cases, the linkage disequilibria are systematic, that is to say, of the same direction in all populations studied: (e) Between *Adh* and chromosome *U* inversions (No. 76). In our case only the Sussex population has the rare allele *1.67* always associated with U_{ST}, but in previous work when this rare

TABLE 5

Tests for random associations between genes and gene arrangements of the same chromosome

			Population	χ^2	d.f.	*P*
1. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	SU	26.673	2	<0.001
2. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	PA	18.839	2	<0.001
3. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4})	PR	14.990	1	<0.001
4. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	BA	15.916	2	<0.001
5. *Lap* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	SU	28.610	2	<0.001
6. *Lap* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	PA	25.241	2	<0.001
7. *Lap* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4})	PR	19.271	1	<0.001
8. *Lap* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	BA	23.226	2	<0.001
9. *Acph* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	SU	0.274	2	0.80–0.90
10. *Acph* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	PA	6.974	2	0.02–0.05
11. *Acph* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4})	PR	0.002	1	0.95–0.98
12. *Acph* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	BA	12.784	2	<0.01
13. *Est-5* (*0.90*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PA	0.975	3	0.80–0.90
14. *Est-5* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PR	1.812	3	0.50–0.70
15. *Est-5* (*0.90*, r)	*versus*	*O* chr. (II_{ST},II_r,II_r)	BA	1.129	2	0.50–0.70
16. *AO* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PA	8.592	3	0.02–0.05
17. *AO* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PR	2.489	3	0.30–0.50
18. *AO* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r,II_r)	BA	3.416	2	0.10–0.20
19. *Xdh* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PA	4.548	3	0.20–0.30
20. *Xdh* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PR	7.226	3	0.05–0.10
21. *Xdh* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r,II_r)	BA	9.146	2	0.01–0.02
22. *ME* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_1,II_{22},II_r)	PA			*1.000
23. *ME* (*1.00*, r	*versus*	*O* chr. (II_{ST},II_r)	PR	0.042	1	0.80–0.90
24. *ME* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	BA			*0.190
25. *Odh* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PR			*1.000
26. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	SU	0.302	1	0.50–0.70
27. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PA	8.589	1	<0.01
28. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r:all within I_{3+4})	PA	4.441	1	0.02–0.05
29. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PR	1.500	1	0.20–0.30
30. *Pept-1* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	BA	0.440	1	0.50–0.70
31. *Lap* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	SU	1.120	1	0.20–0.30
32. *Lap* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PA	0.470	1	0.30–0.50
33. *Lap* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PR	5.547	1	0.01–0.02
34. *Lap* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r:all within I_{3+4})	PR	0.658	1	0.30–0.50
35. *Lap* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	BA	2.320	1	0.10–0.20
36. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	SU			*0.053
37. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r:all within I_{3+4})	SU			*0.063
38. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PA	7.353	1	<0.01
39. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r:all within I_{3+4})	PA	1.833	1	0.10–0.20
40. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	PR	0.030	1	0.80–0.90
41. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r)	BA			*0.027
42. *Acph* (*1.00*, r)	*versus*	*O* chr. (II_{ST},II_r:all within I_{3+4})	BA			*0.074
43. *Est-5* (*0.90*, r)	*versus*	*O* chr. (I_{3+4},I_r)	SU	0.460	1	0.30–0.50
44. *Est-5* (*0.90*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PA	2.990	1	0.05–0.10
45. *Est-5* (*0.90*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PR	0.230	1	0.50–0.70
46. *Est-5* (*0.90*, r)	*versus*	*O* chr. (I_{3+4},I_r)	BA	2.600	1	0.10–0.20

TABLE 5—Continued

			Population	χ^2	d.f.	P
47. *AO* (*1.00*, r)	*versus*	*O* chr. (I_{3+},I_r)	SU	0.010	1	0.90–0.95
48. *AO* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PA	0.090	1	0.70–0.80
49. *AO* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PR	1.870	1	0.10–0.20
50. *AO* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	BA	0.780	1	0.30–0.50
51. *Xdh* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	SU	0.360	1	0.50–0.70
52. *Xdh* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PA	0.280	1	0.50–0.70
53. *Xdh* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PR	1.530	1	0.20–0.30
54. *Xdh* (*1.00*, r)	*versus*	*O* chr. (I_{ST},I_{3+4},I_{3+4+8})	BA	4.223	2	0.10–0.20
55. *ME* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	SU	0.953	1	0.30–0.50
56. *ME* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PA			*0.117
57. *ME* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	PR			*0.063
58. *ME* (*1.00*, r)	*versus*	*O* chr. (I_{3+4},I_r)	BA			*0.046
59. *Aph* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	SU	0.031	1	0.80–0.90
60. *Aph* (*1.00*, 0.73)	*versus*	*J* chr. (J_1,J_{ST})	PA	0.933	1	0.30–0.50
61. *Aph* (*0.73*, r)	*versus*	*J* chr. (J_1,J_{ST})	PR	0.869	1	0.30–0.50
62. *Aph* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	BA	3.004	1	0.05–0.10
63. *Est-7* (*1.04*, 1.00)	*versus*	*J* chr. (J_1,J_{ST})	SU			*0.746
64. *Est-7* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	PA			*0.379
65. *Est-7* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	PR			*0.131
66. *Est-7* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	BA			*0.227
67. *Est-3* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	SU	0.158	1	0.50–0.70
68. *Est-3* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	PA	2.111	1	0.10–0.20
69. *Est-3* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	PR	0.394	1	0.50–0.70
70. *Est-3* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	BA	1.921	1	0.10–0.20
71. *Pgm* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	SU	0.552	1	0.30–0.50
72. *Pgm* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	PA			*1.000
73. *Pgm* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	PR			*1.000
74. *Pgm* (*1.00*, r)	*versus*	*J* chr. (J_1,J_{ST})	BA			*0.366
75. *Idh* (*1.17*, 1.00)	*versus*	*J* chr. (J_1,J_{ST})	SU			*1.000
76. *Adh* (*1.67*, 1.00)	*versus*	*U* chr. (U_{ST},U_r)	SU			*0.050
77. *Hk* (*1.00*, *0.73*, r)	*versus*	*E* chr. (E_{ST},E_{1+2})	SU	1.926	2	0.30–0.50
78. *Hk* (*1.27*, *1.00*, 0.73)	*versus*	*E* chr. (E_{ST},E_8,E_r)	PA	8.013	4	0.05–0.10
79. *Hk* (*1.27*, *1.00*, 0.73)	*versus*	*E* chr. (E_{ST},E_8,E_r)	PR	1.697	4	0.70–0.80
80. *Hk* (*1.27*, r)	*versus*	*E* chr. (E_{ST},E_8,E_{1+2},E_{1+2+9},E_r)	BA	7.537	4	0.10–0.20
81. *Est-9* (*35*, *4*, *34*, *5*, r)	*versus*	*E* chr. (E_{ST},E_{1+2})	SU	5.058	2	0.20–0.30
82. *Est-9* (*34*, *24*, *25*, *35*, r)	*versus*	*E* chr. (E_8,E_{ST}+E_{1+2},E_r)	PA	59.021	8	<0.001
83. *Est-9* (*34*, *36*, *35*, r)	*versus*	*E* chr. (E_8,E_{ST}+E_{1+2})	PA	11.306	3	0.01–0.02
84. *Est-9* (*34*, *4*, *24*, *25*, r)	*versus*	*E* chr. (E_8,E_{ST}+E_{1+2},E_r)	PR	92.687	8	<0.001
85. *Est-9* (*34*, *4*,*35*, r)	*versus*	*E* chr. (E_8,E_{ST}+E_{1+2})	PR	8.258	3	0.02–0.05
86. *Est-9* (*34*, *25*, *35*, *4*, r)	*versus*	*E* chr. (E_{ST}+E_8+E_{1+2},E_r)	BA	57.653	4	<0.001
87. *Est-9* (*34*, *4*, r)	*versus*	*E* chr. (E_{ST}+E_{1+2}(E_8)	BA	6.555	2	0.02–0.05
88. *6-PGD* (*1.00*, r)	*versus*	*A* chr. (A_{ST},A_r)	PR			*1.000
89. *6-PGD* (*1.00*, r)	*versus*	*A* chr. (A_{ST},A_r)	BA			*0.003
90. *G-6-PD* (*1.00*, r)	*versus*	*A* chr. (A_{ST},A_r)	PR			*1.000

SU, PA, PR and BA are symbols for the Sussex, Mt. Parnes, Preveza and Barcelona populations. (Further explanation in the text.)

Probabilities marked with an asterisk (to their left) are Fisher's exact probabilities.

allele was found in other populations (*e.g.*, Mt. Parnes, Kalambaka in Epirus) it was always found in U_{ST}, a rather rare gene arrangement. *Adh* is located within inversions of the U_{1+2} phylad. (f) Between *Est-9* configurations and chromosome *E* inversions (Nos. 82, 83, 84, 85, 86 and 87). As Table 3 also shows, the E_{1+2+9} phylad is associated with configurations 24, 1′4, 25, 1′5, 26, 27, 2′4, etc., not found (or rarely found) in E_8, E_{ST} or E_{1+2}. Also, E_8 is associated with 36, which is rarely found in E_{ST} and E_{1+2}. *Est-9* is located outside of E_{1+2} but within E_{1+2+9}, $E_{1+2+9+12}$ or $E_{1+2+9+3}$, and E_8. Thus, there is no disequilibrium when E_{ST} and E_{1+2} are the only arrangements tested (No. 81), which in fact do not differ in their *Est-9* gene content. These disequilibria are not only found in this study, but have also been described in all other populations studied previously (including earlier samples from Mt. Parnes, Crete, Finland, Austria and Israel) (LOUKAS and KRIMBAS 1975). (g) Between *Pept-1* and gene arrangements of segment I of chromosome *O* (Nos. 1, 2, 3 and 4). *Pept-1* is located within these inversions; thus, O_{3+4} is associated more often than expected with $Pept\text{-}1^{0.40}$ and O_{ST} with $Pept\text{-}1^{1.00}$. O_{3+4+8}, which derives by a simple overlapping inversion from O_{3+4} seems to resemble to O_{3+4} in Sussex and O_{ST} in Mt. Parnes and Barcelona although not in a statistically significant way. In Crete (unpublished data) it does resemble O_{ST} in a statistically significant way. (h) Between *Lap* and gene arrangements of segment I of chromosome *O* (Nos. 5, 6, 7 and 8). *Lap* is located within these inversions. O_{3+4} and O_{3+4+8} are associated with $Lap^{1.00}$ more often than expected.

All the genes that show strong and consistent linkage disequilibria with inversions in all populations studied are located within these inversions (*Pept-1*, *Lap*, *Est-9*). The reverse is not true: genes located within inversions do not necessarily show linkage disequilibria with them. Thus, (i) *Acph* is located within the inversions of segment I of chromosome *O*, as are *Pept-1* and *Lap*, but it does not show linkage disequilibrium with these inversions in the Sussex and Preveza populations (No. 9 and 11). It does in the Mt. Parnes population (No. 10) (where $Acph^{1.00}$ is associated with O_{ST}) and in the Barcelona population (No. 12, where $Acph^{1.00}$ is associated with O_{3+4} and O_{3+4+8}, that is to say, in the contrary direction). Our unpublished results indicate that the Crete population resembles the Barcelona population. *Lap*, *Pept-1* and *Acph* associations with inversions are shown in Table 6. (j) *Hk* is located within the inversions of chromosome *E*. In the tests of Table 5 (Nos. 77, 78, 79 and 80), where all gene arrangements are examined at the same time, *Hk* alleles do not seem to show linkage disequilibrium with these gene arrangements in any of the populations studied. This is also true for the populations of Finland, Austria, Israel, Crete and an earlier sample from Mt. Parnes reported by LOUKAS and KRIMBAS (1975). A closer look at the E_8 gene arrangement shows that it differs in its gene content from E_{ST} in Mt. Parnes and Barcelona, but not E_{ST} in Preveza (in only these three populations is E_8 common) ($\chi^2 = 7.904$, 2 d.f., $0.01 < P < 0.02$ for Mt. Parnes, $\chi^2 = 6.89$, 2 d.f., $0.02 < P < 0.05$ for Barcelona and $\chi^2 = 1.01$, 2 d.f., $0.50 < P < 0.70$ *for* Preveza). As we can see in Table 7, these disequilibria are in different directions in the Mt. Parnes and Barcelona populations ($Hk^{1.00}$ has

TABLE 6

Associations between Pept, Lap *and* Acph *alleles with* O *gene arrangements in all populations studied*

	O_{ST}**				O_{3+4}*				O_{3+4+8}				$O_{3+4+16+2}$			
Gene	SU	PA	PR	BA	SU	PA	PR	BA	SU	PA	PR	BA	SU	PA	PR	BA
Pept-1																
1.60	3	1	1	1	3	1	2	0	1	0	0	1	0	0	0	0
1.00	58	14	22	30	25	30	39	24	8	4	1	11	0	0	0	0
0.40	27	9	16	25	60	116	117	80	15	5	3	14	0	2	1	1
Lap																
1.25	2	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0
1.18	11	0	2	4	0	0	2	0	1	0	0	1	0	0	0	0
1.11	9	5	4	13	3	8	13	6	0	0	0	1	0	0	0	0
1.06	16	6	10	10	4	5	11	11	2	1	2	1	0	0	0	0
1.00	46	12	18	25	77	139	127	80	20	8	1	23	0	2	1	1
0.92	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
0.86	4	0	0	3	4	3	4	6	1	0	1	0	0	0	0	0
0.69	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0
Acph																
2.00	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0
1.88	4	0	0	5	4	7	9	2	0	0	0	0	0	2	0	1
1.00	76	24	31	41	74	129	125	96	21	9	4	25	0	0	1	0
0.54	5	0	7	5	9	19	22	6	3	0	0	1	0	0	0	0
0.25	2	0	1	4	0	0	1	0	0	0	0	0	0	0	0	0

* Indicates that gene arrangements 3+4+1, 3+4+7, 3+4+2, 3+4+22 and 3+4+6, are included in 3+4, since inversions 1, 7, 2, 22, 6 do not overlap 3+4. In a similar way, the gene arrangements O_6 and O_7 are included in O_{ST} (two asterisks). In each case the number of alleles observed is indicated.

a higher frequency in E_8 than E_{ST} in Mt. Parnes, while the contrary is found in Barcelona). For E_{1+2+9} and other arrangements, no disequilibria are detectable. (k) Finally, *Est-7* is probably located within inversion J_1 but does not show any linkage disequilibrium with it (Nos. 63, 64, 65 and 66). The same is true for *ME* and *Odh* and inversions of segment II of chromosome *O*.

The strong and consistent associations in all populations studied of genes and the inversions within which they are located can be explained by selection, but also by a mechanical association of historical origin. Since we believe that every inversion originated by a unique event, we can easily visualize that some alleles have been tied up with the inversions and that not enough time has elapsed for disturbing processes (e.g. mutations) to produce a steady-state equilibrium. It becomes more difficult, however, to sustain this last thesis when other genes located within the *same* inversions show linkage equilibria with them. This is the case for *Hk*, *Est-9* and chromosome E inversions and also (partly at least) for *Acph* on the one hand, and *Pept-1* and *Lap* on the other with chromosome *O* inversions of segment I. *Acph* seems to have reached linkage equilibrium in some populations and not in others; but within these last we can find populations with linkage disequilibria of contrary directions.

TABLE 7

Number of chromosomes bearing different Hk *alleles and belonging to different gene arrangements in the four populations studied*

Hk alleles	E_S	E_{ST}	Inversions E_{1+2}	E_{1+2+9}	$E_{1+2+9+12}$	$E_{1+2+9+3}$
Sussex						
1.43	0	1	1	0	0	0
1.27	1	53	29	0	2	0
1.00	0	49	35	0	2	0
0.73	0	19	7	0	1	0
Mt. Parnes						
1.43	0	0	0	1	0	0
1.27	15	14	7	38	4	0
1.00	27	5	6	51	7	0
0.73	4	3	1	11	1	0
Preveza						
1.27	17	17	3	32	1	0
1.00	25	22	7	54	3	0
0.73	4	7	1	12	0	0
Barcelona						
1.27	10	17	15	17	17	2
1.00	2	24	19	20	22	3
0.73	1	3	2	4	8	0

The existence of a very strong linkage disequilibrium between inversions of segment I of chromosome *O* and those of segment II generates four other cases of linkage disequilibrium between genes located in segment I (and displaying linkage disequilibria with its inversions) and inversions found in segment II. This is the case of *Acph* and segment II inversions in the Barcelona and Mt. Parnes populations (No. 41, 38), of *Lap* and segment II inversions in the Preveza population (No. 33) and finally of *Pept-1* and segment II inversions in the Mt. Parnes population (No. 27). The significance of these tests disappears when the

TABLE 8

Effective distances (in cM) between two genes of chromosome O *for the four populations studied*

	Sussex	Mt. Parnes	Preveza	Barcelona
Est-5–Odh	0.151	0.089	0.083	0.093
Est-5–Ao	0.175	0.102	0.095	0.108
Est-5–ME	0.296	0.168	0.155	0.180
Est-5–Xdh	0.316	0.178	0.164	0.191
Odh–Ao	0.024	0.013	0.012	0.015
Odh–ME	0.145	0.079	0.072	0.087
Odh–Xdh	0.165	0.089	0.081	0.098
ME–Xdh	0.020	0.010	0.009	0.011
Ao–ME	0.121	0.066	0.060	0.072
Ao–Xdh	0.141	0.076	0.069	0.083

test is performed within the same gene arrangement structure of segment I, namely O_{3+4}, (No. 42, 39, 34), or is much reduced (No. 28).

The "middle gene" explanation: It has been suggested to us that genes such as *Hk* and *Acph*, having reached linkage equilibria, would be located toward the middle of the inpersion, while those displaying disequilibria, such as *Est-9*, *Lap* and *Pept-1*, should be found nearer to the inversion breakpoints.

Let us consider first the case of the genes *Hk* and *Est-9*. As far as we know, *Hk* seems to be located inside inversion E_8 and near its distal breakpoint, at least nearer to it than is *Est-9* (see Figure 1). Nevertheless, E_8 and E_{ST} gene arrangements do not differ for *Hk* alleles in the Preveza population and differ in contrary directions in the Mt. Parnes and Barcelona populations, whereas in all populations E_8 does differ from E_{ST} for *Est-9* configurations (which is contrary to this explanation). Furthermore, the E_{1+2+9} gene arrangement is a complex of three overlapping inversions: E_1 (absent now from natural populations), E_2 (always on E_1 in the E_{1+2} gene arrangement) and E_9 (always on the E_{1+2} gene arrangement, thus forming E_{1+2+9}). E_{1+2+9} includes *Hk* and *Est-9*, but cannot easily exchange either with the E_{ST} gene arrangement. On the contrary, E_{1+2+9} differs by a big single inversion (E_9) from E_{1+2}. *Hk* is located slightly nearer the middle of this gene arrangement than is *Est-9*. This, according to the above-mentioned hypothesis, would imply that E_{1+2+9} and E_{1+2} should be the same for *Hk* alleles (which is the case) but different from E_{ST} (which is not the case). For the *Est-9* gene, it would also imply that the E_{1+2+9} gene arrangement should be different from E_{1+2} (which is the case). As E_{1+2} does not include this gene, it should not differ from E_{ST} (which is also the case). Thus the middle-gene hypothesis cannot satisfactorily explain all our data.

In order to consider the case for *Pept-1*, *Lap* and *Acph*, we need to know their exact positions. Unfortunately, a lack of markers between them (from *ch*

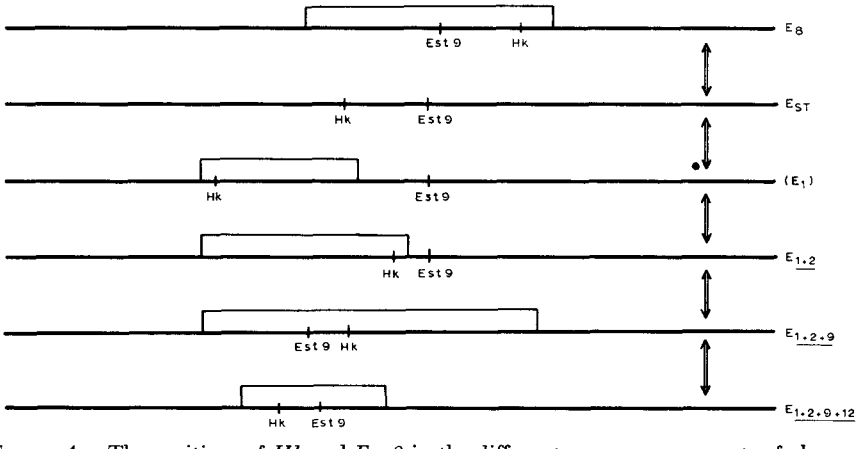

FIGURE 1.—The positions of *Hk* and *Est-9* in the different gene arrangements of chromosome *E*. Double arrows indicate the only possible simple paths for the phylogeny of these gene arrangements. The existence of the E_1 gene arrangement has been postulated; it was never observed in natural populations.

to the distal end of the chromosome) does not permit such a direct knowledge. However, we do know the following (LOUKAS, *et al.* 1979):

(a) *Pept-1*, *Lap* and *Acph* are within the overlapping inversions O_{3+4} (from crosses of O_{3+4}/O_{ST} females).

(b) These three genes are within inversion 16 (from crosses of O_{3+4+16}/O_{3+4} females). Inversion 16 overlaps 3+4 and is on it.

(c) The distances (from uncorrected recombination values) and order of genes on the O_{3+4} gene arrangement are *Pept-1*—27.8 cM—*Lap*—14.1 cM—*Acph*. We do not know whether *Pept* or *Acph* is near the distal end of the chromosome.

(d) We know that in the O_{ST} gene arrangement the distance between *Lap* and *Acph* is equal to 12.9 cM, and that both these genes are very far away (nearly 50 cM) from *Pept-1*.

Luckily, there is only one topological solution to this problem, shown in

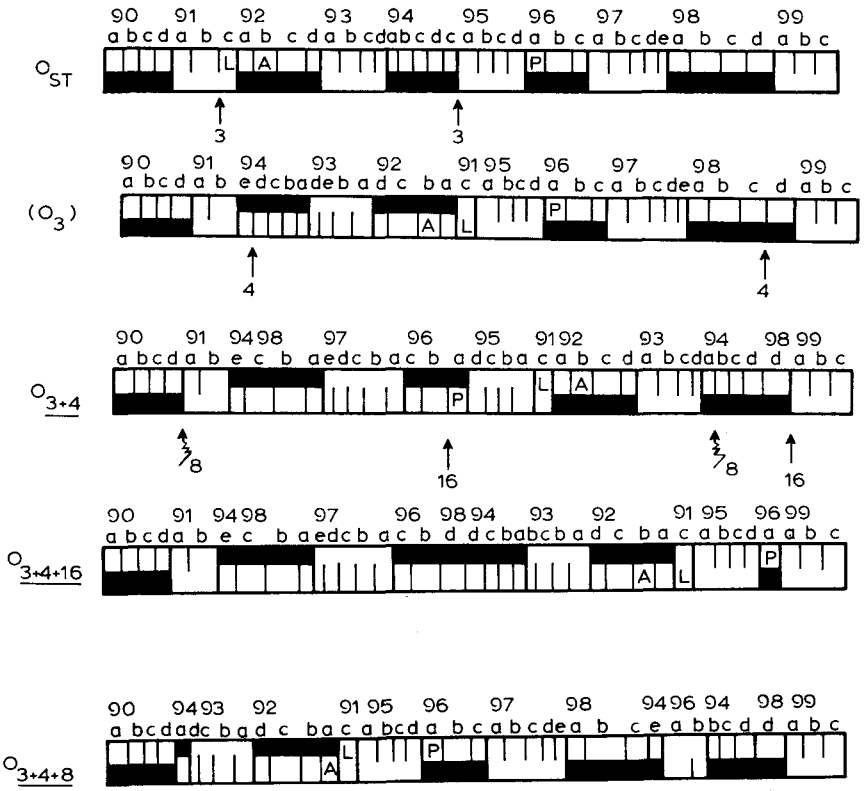

FIGURE 2.—Different gene arrangements of chromosome *O* (only the distal portion of this chromosome is indicated according to the map of KUNZE-MÜHL and MÜLLER 1958). Overlapping inversions are indicated by a continuous line under the numbers indicating the inversions. L, A and P indicate the approximate positions of *Lap*, *Acph* and *Pept-1* in the different gene arrangements.

Figure 2. In this Figure, we indicate the approximate positions of these three genes in the following gene arrangements: O_{ST}, O_3 (a hypothetical arrangement not observed in nature), O_{3+4} (derived from the superimposition of inversion 4 on inversion 3), O_{3+4+16} (derived from the superimposition of inversion 16 on inversions 3+4) and O_{3+4+8} (derived from the superimposition of inversion 8 on 3+4). It does not seem that double crossovers would favor the exchange of gene *Acph* rather than *Lap* or *Pept-1* in the heterozygous females O_{3+4}/O_{ST} or O_{3+4+8}/O_{ST}. Thus, the middle-gene explanation is not satisfactory in this case either.

Are the nonsystematic disequilibria a product of drift? All the associations observed, except for the systematic ones (those of genes *Pept-1*, *Lap* and *Est-9* with the inversion of chromosomes *O* and *E*, respectively, and between genes of *Est-9* complex), cannot be attributed to selection by tight arguments. On the other hand, the question could be reversed: What are the arguments that these associations are a product of genetic drift?

Tight arguments for drift also cannot be advanced since it appears possible that selection does not act uniformly in all populations all the time. It is, however, of some interest to estimate the value of the effective population sizes in order to decide whether it is probable that the observed associations could be generated by drift.

It should first be remarked that observed relations between genes are not independent of the distances separating them. However, these are "effective" genetic distances between the pairs of genes forming the couples used in our tests. These "effective" genetic distances take into account the effects of inversion heterozygosity on the frequency of crossing over and have been calculated according to ZOUROS *et al.* (1974). Our data permitted these calculations only for the genes of chromosome *O* (of course *Lap*, *Pept-1* and *Acph* have been excluded from these calculations). These effective distances are indicated in Table 8 and give an idea of the distances between the genes examined for departure from the linkage equilibrium state.

Unfortunately, since allelic frequencies generally depart from 0.50, we cannot use all these data to calculate the effective sizes of populations that could produce such linkage disequilibria [using formulas of SVED (1971) and OHTA and KIMURA (1969)].

A way to approach the problem however, is to use the associations of genes located within inversions with these inversions and, since practically $c=0$, to apply to them the HILL and ROBERTSON (1968) formula:

$$E(D^2) = \frac{1}{15} p(1-p)q(1-q)\{6(1-F)-5(1-F)^3-(1-F)^6\} ,$$

where D is the determinant of linkage disequilibrium. We used *Est-7* with inversion J_1, *Acph* with segment I of chromosome *O* inversions and *Hk-1* with chromosome *E* inversions. These are the results found by a minfun computer

program (we have estimated the mean $p(1-p)q(1-q)$ for each population):

	$\overline{D^2}$	$\overline{p(1-p)q(1-q)}$	F	N_em
Sussex	0.000021	0.0272709	0.00077	324
Mt. Parnes	0.000224	0.0196141	0.01169	21
Preveza	0.000099	0.0415310	0.00240	10
Barcelona	0.000628	0.0233208	0.02852	9

N_em is calculated from $F = \frac{1}{4N_e(m+u)+1}$, simplifying by ignoring u (mutation rate) since it is much smaller than m (migration rate).

Is this estimation of N_em too small?

Effective population size: There are three methods to estimate the effective population size:

(a) By the allelism of lethals. Data of PANOPOULOU (1973 and unpublished observations) give an N_em estimation of 59 for the Mt. Parnes population (and 175 for Crete) using the old method of DOBZHANSKY and WRIGHT (1941).

(b) By the ecological method (based on a number of ecological observations). Thus BEGON (1977) estimated that an English population near Leeds has an effective size of approximately 400 individuals. We do not have such direct estimations for the populations we studied here.

(c) By the temporal method (KRIMBAS and TSAKAS 1971). Using the markers, *Est-3*, *Est-5*, *Est-7*, *Acph*, *Aph*, *Pgm*, *Hk-1*, *ME*, *Xdh* and *Ao*, we have estimated the allelic frequencies in three successive samples from the Mt. Parnes population: one on September 1975, a second on September 1976 and a third on May 1977. The between-samples F was calculated as

$$F_t = \frac{1}{n} \Sigma \frac{(\Delta p)^2}{p_0(1-p_0)} ,$$

where n is the number of alleles, $(\Delta p)^2$ is the squared difference between the frequencies of the same allele in two successive samples t generations apart, and p_0 the frequency in the first sample. This crude estimation of F_t has been corrected by substracting a quantity $n \left[\frac{1}{N_1} + \frac{1}{N_2} \right]$ to take care of the sampling errors due to the restricted size of our samples, where n is the number of genes tested, and N_1 and N_2 are the sample sizes (numbers of genes) used. For $t=7$, between September 1975 and September 1976, we get $N_e=233\pm65$. For $t=2$, between September 1976 and May 1977, the effective size become infinite. These two estimations differ and this subject will be discussed in detail elsewhere (BEGON, KRIMBAS and LOUKAS, in preparation). The first sample points to a small enough size to produce the observed or even greater disequilibria; the second one does not.

DISCUSSION

The results reported in this paper are in good agreement with earlier studies of PRAKASH and LEWONTIN (1968, 1971), PRAKASH (1974, 1976) and PRAKASH

and MERRITT (1972) in *D. pseudoobscura* and *D. persimilis*; of PRAKASH and LEVITAN (1973, 1974) on *D. robusta*; of KOJIMA, GILLESPIE and TOBARI (1970), MUKAI, METTLER and CHIGUSA (1971), CHARLESWORTH and CHARLESWORTH (1973), MUKAI, WATANABE and YAMAGUCHI (1974); LANGLEY, TOBARI and KOJIMA (1974); MUKAI and VOELKER (1977); LANGLEY, ITO and VOELKER (1977) and LANGLEY, SMITH and JOHNSON (1978) in *D. melanogaster*.

Especially the results of ZOUROS *et al.* (1974), LOUKAS and KRIMBAS (1975), CHARLESWORTH, CHARLESWORTH and LOUKAS (1979) and unpublished observations kindly communicated by PREVOSTI and SPERLICH, all in *D. subobscura*, are in full agreement with the present results. The only disagreement was with the report of ZOUROS and KRIMBAS (1973).

All these studies, as well as the larger-scale present one, reveal the paucity of linkage disequilibria between alleles belonging to two different genes. This paucity, in our case, is partly due to the impossibility of detecting all kinds of alleles electrophoretically. As ZOUROS, COLDING and MACKAY (1977) have shown, the effect of combining alleles into electrophoretic classes reduces experimental discrimination considerably, and thus only a portion of the linkage disequilibria is detected. Using temperature sensitivity together with electrophoretic mobility for chromosome *O* genes does not seem to lead to the detection of a greater number of linkage disequilibria (work in progress). Thus, it is possible that the paucity of such linkage disequilibria is genuine, and we are tempted to conclude that epistatic models are not the rule (*e.g.*, those of SVED, REED and BODMER 1967 or KING 1967); the chromosome would not be organized as a unit of selection, as FRANKLIN and LEWONTIN (1970) proposed.

However, the existence of linkage disequilibria also depends on genetic distances. It is true that, as shown in Table 8, the distances between pairs of genes is considerable, although for some it is rather small (*e.g.*, *Odh* and *Ao*). When the distances become much smaller, however, as between genes of the *Est-9* complex, then quite a few such disequilibria are observed. We still do not know whether this is a general rule or a special characteristic of *Est-9* genes.

The cases of linkage disequilibria between genes and inversions could be classified as cases of nearly zero genetic distance. Observed linkage disequilibria could be due to a number of causes such as, (a) the grouping of individuals belonging to two or more different panmictic units, if the allelic frequencies of two genes are correlated. Then the determinant D of linkage disequilibrium is equal to the covariance of the frequencies:

$$\mathrm{cov} p_A q_B = \frac{\Sigma p_A q_B}{n} - \bar{p}_A \bar{q}_B = g_{AB} - \bar{p}_A \bar{p}_B = D \ ,$$

where g_{AB} is the frequency of the gamete bearing *AB*. Care was taken in Sussex, Mt. Parnes and Preveza samplings (which we personally sampled) to take the animals from a very restricted area, usually from only one trap under one tree, in order to avoid population heterogeneity. The spurious association between *Lap* and *Pept-1* alleles in the Sussex population could be described as an effect of this kind if O_{ST} and $O_{\underline{3+4}}$ chromosomes were considered formally to belong to

two different "populations". (b) Sampling errors. Most of the systematic linkage disequilibria presented in this study can not possibly be due to sampling errors; the same direction of linkage disequilibria was found in all four populations studied by us and in other populations studied by other anthors. (c) Historical factors and drift. These are transient associations; however, populations can keep these associations for a long time, depending on the magnitude of the disturbing processes. One such process is double crossing over, which has been examined in detail previously; it cannot explain satisfactorilly why genes such as *Hk* are in linkage equilibrium while others such as *Est-9* are not (although the "allelic" discrimination is much better for *Est-9* than for *Hk*). The other process is mutation. To consider this matter, we need to know the age of these inversions. Only for the case of Hawaiian Drosophila species do we know with certainty their age (1–2 million years). For *Drosophila subobscura*, we could equally well argue that they originated 10,000 to 15,000 years ago or even millions since the Meiocene (MS in preparation). Thus, age of inversions cannot help us in this case. (d) Epistatic selection, which remains a real possibility—and is the most plausible explanation for *Lap, Pept-1* and *Est-9* and inversions. The linkage disequilibria between genes of the *Est-9* complex can also be attributed to selection, with some degree of certainty.

The main support for selection underlying the associations of *Lap, Pept-1* and *Est-9* with inversions comes from examination of the middle-gene explanation. A similar argument can be advanced; it is supposed that an inversion frequently may have differentiated by acquiring a new allele not found in the original gene arrangement. By drift, this allele increased in frequency, *e.g.*, $Hk^{1.27}$ in E_8 towards E_{ST} in Barcelona. But if this is so, populations should also drift for gene frequencies (although to a much lesser degree). What seems astonishing is the homogeneity in allelic frequencies of all populations studied (except for the genes displaying linkage disequilibrium with inversions).

Effective population size estimations by the temporal method turn out to be rather small. This method could give reliable results if the genes used were neutral. We tried to use genes for which we had no indications of linkage disequilibria of a possible selective origin. It is reassuring that estimations by pure ecological methods turn out to be of the same order of magnitude. Work on this subject is in progress and will permit us to evaluate the accuracy of the estimations of N_e by different methods.

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Corresponding editor: W. W. ANDERSON