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Linkage Disequilibrium in a Local Population of *Drosophila melanogaster**

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ABSTRACT **461 second chromosomes of *Drosophila melanogaster* were extracted from a Raleigh, N.C. population and four enzymes controlled by the genes located in this chromosome (alcohol dehydrogenase (EC 1.1.1.1.), malate dehydrogenase-1 (EC 1.1.1.37), glycerol-3-phosphate dehydrogenase-1 (EC 1.1.1.8), and α-amylase (EC 3.2.1.1), were assayed electrophoretically and cytologically (salivary-gland chromosomes). Linkage disequilibrium could not be detected among any pair of isozyme genes, except in one case that is best explained as due to a chance error in estimation. Some disequilibria were detected, however, between isozyme genes and polymorphic inversions. The relative viabilities of homozygous and heterozygous combinations of these chromosomes were estimated with respect to the alcohol dehydrogenase alleles and the glycerol-3-phosphate dehydrogenase alleles; no significant difference could be detected. The role of epistasis in natural populations is discussed on the basis of these results.**

Since Lewontin and Hubby (1) first reported that a large proportion of isozyme loci are polymorphic in *Drosophila pseudoobscura* populations, appreciable supporting data have been accumulated from many organisms (2, 3), including man (4); the phenomenon is probably a manifestation of a general rule. However, how these polymorphisms are maintained is not completely understood. Prakash *et al.* (5) have concluded on the basis of extensive surveys of polymorphisms in several natural populations of *D. pseudoobscura* that "the most likely explanation for the maintenance of this genic variation is some form of balancing selection".

If balancing selection is indeed involved, it should be of a type that does not create a large genetic load. It also follows from basic theory that if overdominance is involved, then some amount of epistasis is necessary to reduce the load. Although nonrandom associations between certain alleles and specific inversions have been discovered for the α-amylase (EC 3.2.1.1) locus and a larval-protein locus (*Pt-10*) in *D. pseudoobscura* and *D. persimilis* (6), there has been no clear evidence of any interaction between two or more isozyme genes.

Certain mathematical models that may satisfy the above condition (that of reduced segregation load) have been proposed by several investigators (7–10). These authors have assumed overdominance and a threshold or truncation selection (which can be considered equivalent to an extreme form of epistasis). Wills *et al.* (10) have shown on the basis of computer simulation experiments that a large number of polymorphisms can be maintained together with a considerable magnitude of linkage disequilibrium, even between relatively distant genes, under the condition of a 5–20% elimination of individuals that have the lowest fitness values, if heterozygote superiority and linkage is assumed. In fact, Lewontin (11) emphasized several years ago that epistasis is required in order for linkage to be important in natural selection. He also predicted the existence of linkage disequilibrium in random mating populations from his simulation experiments.

For the above reasons, it is necessary to examine whether or not linkage disequilibria between isozyme genes truly exist in natural populations. Accordingly, we have extracted second chromosomes from *D. melanogaster* collected in a state park near Raleigh, N. C., assayed the enzymes that are controlled by the genes located in the second chromosomes, and examined the salivary gland chromosomes. The degrees of linkage disequilibria between isozyme genes, as well as between isozyme genes and polymorphic inversions, have been estimated. The preliminary results have been published (12).

MATERIALS AND METHODS

315 and 146 second chromosomes were extracted in the summers of 1968 and 1969, respectively, using the *Cy-Pm* inversion method. The chromosome lines were maintained at 19°C. Every chromosome was maintained balanced (no recombination) with SM1(*Cy*) chromosome, which helped to maintain the less viable or lethal chromosomes. These chromosomes were assayed with respect to isozyme variation and inversions.

The following four enzyme loci, which are known to be located in the second chromosome (13, 14), were examined: glycerol-3-phosphate dehydrogenase-1 (*Gpdh-1* [EC 1.1.1.8]; map position is 17.8), malate dehydrogenase-1, (*Mdh-1* [EC 1.1.1.37], map position is 35.3), alcohol dehydrogenase (*Adh* [EC 1.1.1.1], map position is 50.1), and α-amylase (*Amy*, map position is 77.3). The three dehydrogenases were assayed by starch-gel electrophoresis (15), while α-amylase was assayed by acrylamide-gel electrophoresis (5).

For the materials collected in 1969, the degrees of dominance of viability genes were estimated by the *Cy* method (16). The viability values of random heterozygotes, as well as homozygotes, were estimated. The viabilities of 288 random heterozygotes, using 145 independent chromosomes, were used for the estimation of relative viabilities of homozygotes and heterozygotes with respect to the isozyme genes. This estimation was possible because each chromosome was assayed for the enzymes described above.

RESULTS

The *Adh*, *Mdh-1*, and *Gpdh-1* loci were found to be heteroallelic for the known alternative alleles *Fast* (*F*) and *Slow* (*S*)

* Since writing the present manuscript, we have learned that a very similar work on linkage disequilibria between genes and inversions has been reported by Kojima *et al.* (26).

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TABLE 1. *Gene frequencies of Fast (F) alleles*

Year	Enzyme			
	Adh	*Gpdh-1*	*Mdh-1*	*Amy**
1968	0.260 ± 0.0247	0.825 ± 0.0214	0.032 ± 0.0099	0.879 ± 0.0184
1969	0.349 ± 0.0395	0.788 ± 0.0338	0.021 ± 0.0117	0.863 ± 0.0285

Adh: alcohol dehydrogenase.
Gpdh-1: glycerol-3-phosphate dehydrogenase-1.
Mdh-1: malate dehydrogenase-1.
Amy: α-amylase.
* Stands for the Amy^1 allele.

(13), while the *Amy* locus was polymorphic for the three alleles Amy^1, $Amy^{1,3}$, and $Amy^{1,6}$ in the 1968 samples and the two alleles Amy^1 and $Amy^{1,3}$ in the 1969 samples, although more than three alleles have been reported for this locus (14). Amy^1 is the most frequent allele; it will be called *F*, with all other alleles called *S*.

Gene frequency

The gene frequencies at each locus examined in 1968 and 1969 samples are given in Table 1. There are no significant differences between the 2 years, indicating that the frequencies of isozyme alleles are fairly *stable over years.*

Linkage disequilibrium

The linkage relationship between any two of the four loci studied, considering all chromosomes assayed, is presented in Table 2. Linkage disequilibrium was examined using the χ^2 test. Even in the cases where one of the expectations shows very low frequency, the χ^2 test (17) was performed. Only one case (*Mdh-1* and *Amy* in 1968) out of 12 was

TABLE 2. *Gametic array for any two loci of the four, the Adh, Gpdh-1, Mdh-1, and Amy loci (values in parentheses refer to inversion-free chromosomes)*

Loci	Year	Gametic array*				Total	χ^2	*D*	*R*
		FF	*FS*	*SF*	*SS*				
Adh	1968	67	15	193	40	315	0.053	−0.0021	−0.0130
versus		(63)	(13)	(145)	(31)	(252)	(0.010)	(0.0010)	(0.0062)
Gpdh-1	1969	38	13	77	18	146	0.849	−0.0148	−0.0763
		(36)	(13)	(61)	(16)	(126)	(0.342)	(−0.0137)	(−0.0667)
Adh	1968	3	79	7	226	315	0.084	0.0013	0.0164
versus		(3)	(73)	(6)	(170)	(252)	(0.045)	(0.0011)	(0.0133)
Mdh-1	1969	2	49	1	94	146	1.357	0.0065	0.0964
		(2)	(47)	(1)	(76)	(126)	(0.998)	(0.0066)	(0.0890)
Adh	1968	71	11	205	27	314	0.180	−0.0034	−0.0239
versus		(65)	(11)	(152)	(24)	(252)	(0.031)	(−0.0018)	(−0.0111)
Amy	1969	46	5	80	15	146	1.006	0.0136	0.0830
		(44)	(5)	(62)	(15)	(126)	(1.930)	(0.0220)	(0.1238)
Gpdh-1	1968	10	250	0	55	315	2.184	0.0055	0.0833
versus		(9)	(199)	(0)	(44)	(252)	(1.974)	(−0.0087)	(0.1169)
Mdh-1	1969	2	113	1	30	146	0.268	−0.0025	−0.0429
		(2)	(95)	(1)	(28)	(126)	(0.185)	(−0.0024)	(−0.0383)
Gpdh-1	1968	229	30	47	8	314	0.374	0.0043	0.0345
versus		(181)	(27)	(36)	(8)	(252)	(0.821)	(0.0075)	(0.0571)
Amy	1969	99	16	27	4	146	0.021	−0.0017	−0.0120
		(81)	(16)	(25)	(4)	(126)	(0.122)	(−0.0048)	(−0.0311)
Mdh-1	1968	6	4	270	34	314	7.557†	−0.0088	−0.1551
versus		(5)	(4)	(212)	(31)	(252)	(7.286)†	(−0.0109)	(−0.1700)
Amy	1969	2	1	124	19	146	0.999	−0.0040	−0.0827
		(2)	(1)	(104)	(19)	(126)	(0.702)	(−0.0042)	(−0.0746)

D stands for the index of linkage disequilibrium.
R stands for the correlation of gene frequencies.
* The two alleles of each gametic array correspond, respectively, to loci under consideration.
† Significant at the 1% level.

significant. Even in this case, significant linkage disequilibrium disappears if the frequency of *FS* chromosomes (*F* in *Mdh-1* and *S* in *Amy*) decreases from 4 to 2. In addition, significant linkage disequilibrium was not found in 1969. Thus, the disequilibrium in 1968 might be due to chance in the process of estimation and, therefore, will be disregarded in the discussion. The magnitude of linkage disequilibrium (D, ref. 18) and the correlation of gene frequencies (19) ($R = D/\sqrt{p(1-p)\,q(1-q)}$: p and q are the frequencies of F alleles in respective loci) were calculated (Table 2).

Inversion-carrying chromosomes were detected at a frequency of 0.20 (63 out of 315) in 1968 and 0.14 (20 out of 146) in 1969. Of these, Inversion A (the breakage points are approximately 51D and 57A, and might be the same as *In(2R)NS*(14) and Inversion C (the breakage points are approximately 22D and 33F, the same as *In(2L)Cy*, but not associated with the *Cy* gene(14) were polymorphic in both years, and their frequencies were relatively high, i.e., Inversion A: 30/315 in 1968 and 14/146 in 1969, and Inversion C: 14/315 in 1968 and 3/146 in 1969. The nonrandom associations between the isozyme genes and these two polymorphic inversions were examined. The results are presented in Table 3. Significantly nonrandom associations between isozyme alleles and inversions were detected in 1968 between Inversion A and *Adh*, Inversion A and *Amy*, and Inversion C and *Adh*, although none was found in 1969, probably due to a relatively small sample size (in fact, the R values did not change significantly).

Simultaneous tests were conducted for the three combinations above of inversions and isozyme genes, and for Inversion C versus *Gpdh-1*. In Inversion A versus *Adh*, the relationship $z \doteq \Sigma\chi/\sqrt{n}$ was employed, where n is the number of χ's. The z value is 2.692, which indicates nonrandom association ($P < 0.01$). A joint probability was calculated for Inversion A versus *Amy*. We have assayed amylase for 29 Inversion A lines in 1968 (except one that was lost) and 14 in 1969. All Inversion A chromosomes carried the *Amy*^{1} (or *F*) allele. If we assume that there is a random association between the inversions and the *Amy* alleles, the probability for the above phenomena become $(0.879)^{29}\ (0.863)^{14} = 0.003$. This is so small that we must reject the hypothesis of random association. The same test was applied in the case of Inversion C versus *Adh* and Inversion C versus *Gpdh-1*. Random association was again rejected against *Adh* ($P = 0.00024$), but not against *Gpdh-1* ($P = 0.090$).

Although the *Gpdh-1* locus is included in Inversion C, significant nonrandom association of the inversion and the alleles was not detected. The location of the *Amy* locus and the *Adh* locus are very close to the breakage points of Inversions A and C, respectively. Thus, recombination is greatly

TABLE 3. *Gametic array for isozyme genes and polymorphic inversions (where I and N refer to inverted and noninverted arrangements, and F and S refer to isozyme alleles)*

Inversion and locus	Year	Gametic array *IF*	*IS*	*NF*	*NS*	Total	χ^2	D	R
Inv. A versus *Adh*	1968	3	27	79	206	315	4.426*	−0.0152	−0.1185
	1969	2	12	49	83	146	2.904	−0.0198	−0.1410
			(Joint Prob. ≤0.01)						
Inv. A versus *Gpdh-1*	1968	23	7	237	48	315	0.793	−0.0056	−0.0502
	1969	12	2	103	29	146	0.460	0.0067	0.0553
Inv. A versus *Mdh-1*	1968	1	29	9	276	315	0.003	0.0002	0.0029
	1969	0	14	3	129	146	0.325	−0.0020	−0.0472
Inv. A versus *Amy*	1968	29	0	247	38	314	4.399*	0.0112	0.1184
	1969	14	0	112	20	146	2.458	0.0131	0.1298
			(Joint Prob. = 0.0030)						
Inv. C versus *Adh*	1968	0	24	82	209	315	9.143†	−0.0198	−0.1704
	1969	0	3	51	92	146	1.644	−0.0072	−0.1061
			(Joint Prob. = 0.00024)						
Inv. C versus *Gpdh-1*	1968	22	2	237	54	315	1.585	0.0072	0.0709
	1969	3	0	112	31	146	0.826	0.0044	0.0752
			(Joint Prob. = 0.090)						
Inv. C versus *Mdh-1*	1968	0	24	10	281	315	0.852	−0.0024	−0.0520
	1969	0	3	3	140	146	0.064	−0.0004	−0.0210
Inv. C versus *Amy*	1968	22	2	254	36	314	0.347	0.0029	0.0332
	1969	3	0	123	20	146	0.486	0.0028	0.0577

* Significant at the 5% level.
† Significant at the 1% level.

TABLE 4. *Relative average viability values of homozygotes and heterozygotes with respect to the Adh locus and Gpdh-1 locus*

Genotype	*Adh*	*Gpdh-1*
FF	1.0093 ± 0.0141 (41)	1.0000 ± 0.0085 (182)
SS	1.0000 ± 0.0103 (137)	1.0418 ± 0.0383 (12)
Pooled homozygotes	1.0022 ± 0.0086 (178)	1.0026 ± 0.0083 (194)
FS	1.0066 ± 0.0104 (110)	1.0184 ± 0.0093 (92)

The average viability value of the homozygotes for the common allele was assumed to be 1.0000.

The figures in parentheses indicate the number of crosses, in each of which eight vials were used (average number of flies counted per cross = 1327.7).

restricted, which tends to maintain any nonrandom association. In the case of Inversion A versus *Adh*, the map distance between the left breakage point of the inversion and the *Adh* locus is about 30 centimorgans (14). However, an inversion suppresses recombination even outside the inversion itself. In the case of Inversion A, the recombination value is reduced to about 1.5% between the centromere and the inversion (14), and the distance between the *Adh* locus and the centromere is very small. Thus, this low frequency of recombination must be one of the contributing factors for the linkage disequilibrium.

Although the *Mdh-1* locus is included in Inversion C, a significant nonrandom association between its alleles and this inversion was not detected. It is difficult to know whether the nonrandom association could not be detected because of the low frequency of the *F* alleles at the *Mdh-1* locus or because the *Mdh-1* locus expresses no significant interaction.

Linkage disequilibrium was tested for any two of the four loci for all inversion-free chromosomes, which numbered 252 in 1968 and 126 in 1969. The data and results are presented in parentheses in Table 2. Again, linkage disequilibrium was detected only for the case of *Mdh-1* and *Amy*.

Next, inversion-carrying chromosomes, excluding Inversions A and C, were examined. A clear conclusion could not be drawn because of the small numbers involved (14 in 1968 and 3 in 1969), but at least the isoallelic frequencies were not significantly different from those of inversion-free chromosomes and linkage disequilibrium could not be detected between any two of the four loci.

Finally, linkage disequilibria among three loci were examined, using all chromosomes. Again the number of chromosomes was not large enough and appropriate tests could not be made except for the case of the *Adh*, *Gpdh-1*, and *Amy* loci, where significant disequilibrium was not detected.

Viability test

Relative viabilities of three genotypes, *FF*, *FS*, and *SS*, were estimated at the *Adh* and *Gpdh-1* loci by use of the data obtained in the estimation of the degrees of dominance of lethal genes and mildly deleterious genes (or viability polygenes). Reliable data could not be obtained for the *Amy* and *Mdh-1* loci because the numbers of heterozygotes were small, due to the very high gene frequency of the common alleles. Six experiments were conducted at different times. In each experiment, the average relative viabilities of the genotypes were determined, with the common-allele homozygotes set at 1.00. The results of all experiments were pooled, being weighted by the numbers of crosses. These data were based on a total count of about 0.4 million individuals and are presented in Table 4. There are no significant differences among the three genotypes in each locus. The numbers of crosses for the homozygotes for the rare alleles are small. Thus, all the homozygote viabilities were pooled (Table 4). In both loci the average heterozygote viabilities are slightly, but not significantly, larger than those of the average homozygotes (0.4% at the *Adh* locus and 1.6% at the *Gpdh-1* locus). Therefore, it may be concluded that the magnitude of heterozygote superiority is essentially nil, or at the most very small, in this case.

DISCUSSION

The significant findings of the present experiment are that the isoallelic frequencies at the four loci examined were similar in two consecutive summer samples, and that all were in approximate linkage equilibrium with each other, including those that showed some nonrandom association with certain natural inversions. Also, the effective size of the population has been estimated by Nei's formula (20) to be about 22,000–24,000 (estimated on the basis of the frequency of lethal-carrying second chromosomes, which was 0.411 in 1968 and 0.359 in 1969, and on the rate of allelism among them, which was 0.0067 in 1968 and 0.0065 in 1969). These findings give no information as to whether the isozyme variations have been maintained by selection or whether the alleles are effectively neutral, but at least it is unlikely that the maintenance results from mass migration between dissimilar gene pools or that strong selection pressures are counteracting random drift.

According to the data of simulation experiments performed by Wills *et al.* (10), an appreciable amount of linkage disequilibrium could be formed between two genes separated from each other by 7% recombination, if overdominance and truncation selection (5%) exist. If we assume that any inversion suppresses recombination completely in the arm where it is located, this distance would correspond to about 16.6% in the present *D. melanogaster* population, where the frequency of inversion-carrying chromosomes is about 20%. Linkage disequilibrium might be formed if there is truncation selection, even though overdominance does not exist (21). Furthermore, computer simulation of a five-locus model (11) showed that a cumulative linkage effect might be expected even for quite-distant genes, if overdominance exists and the degree of interaction is *relatively large*.

In the present experiment, linkage disequilibrium between any two isozyme genes studied was not detected. However, a clear conclusion cannot be drawn since the map distances between adjacent loci (17.5, 14.8, and 27.2) are relatively large (but the smallest value, 14.8, is less than the above 16.6%). At least it can be said that if epistasis exists at all, its magnitude is certainly not large; therefore, it cannot possibly create linkage disequilibrium between two genes about 15 map units apart. Thus, the present data offers no evidence supporting either the overdominance-truncation selection model or the concept of linkage disequilibrium through cumulative linkage effect. Moreover, we have estimated a heritability (broad sense) of viability to be 0.0020 (using 145 2nd chromosomes and counting about 0.2 million flies), which means that even *if* truncation selection is operating at the phenotypic level, the genetic consequences

(the distribution of the genetic values of selected individuals) would be little different from the case of classical, independent-locus selection.

Inversion A and Inversion C were detected as polymorphic ones, the frequencies of which were relatively high. A was first discovered in 1913 and C in 1921 (14); both are now known to be cosmopolitan (14, 22). Although it is not known when or where these inversions first occurred, it is reasonable to suppose that each originated uniquely some time ago. A linkage disequilibrium was detected between the *Adh* locus and Inversion A, though they are located in the different arms. The recombination value between this locus and the inversion is about 6.4%, or effectively 3.2% since there is no crossing over in males. It appears that the frequency of Inversion A is stable (0.0952 in 1968 and 0.0959 in 1969). That the isozyme-inversion linkage disequilibrium is not due to random genetic drift alone can be shown as follows: First, correlation coefficients between the gene frequency and the inversion frequency (R) were calculated to be -0.12 in 1968 and -0.14 in 1969. These absolute values are significantly larger than the value expected on the basis of random genetic drift (23) (approximately 0.019). Second, the D and R values in 1969 are rather larger than that in 1968 at face value (the D value expected in 1969 on the basis of 1968 data is 0.0110; the observed value is 0.0198). It is appropriate to assume some interaction between the *Adh* locus and Inversion A. Hence, selection cannot be rejected.

Linkage disequilibrium was also detected between the *Adh* locus and Inversion C and between the *Amy* locus and Inversion A. These linkage disequilibria might be maintained primarily through the lack of recombination between the inversion and the genes in question. It is difficult to obtain any evidence for interaction, even if it exists. These results are consistent with those reported by Prakash and Lewontin (6). Although the *Gpdh-1* locus occurs within Inversion C, the alleles did not show nonrandom association with the inversion. This was perhaps caused by double crossover and little or no interaction of this gene with adjacent genes. Thus, there is a possibility of intrinsic overdominance of the *Gpdh-1* alleles.

In the present experiment, the minimum distance between isozyme genes for which linkage disequilibrium was shown not to occur was estimated to be about 15 map units. It goes without saying that permanent linkage disequilibrium might exist between isozyme genes that are closer together than those studied here. However, O'Brien *et al.* (24) did not detect a linkage disequilibrium in *D. melanogaster* between the leucine aminopeptidase-D locus and the acid phosphatase-1 locus, which are only about three map units apart (effectively, having 1.5% recombination). Thus, it appears that the magnitude of interaction among isozyme genes, if it exists at all, may be generally very small, and therefore unable to create linkage disequilibrium. This is reasonably speculated also from the result that there is little selection in viability among *FF*, *FS*, and *SS* genotypes at the *Adh* and *Gdph-1* loci. Although the present results do not completely rule out a possibility of linkage disequilibria among extremely-closely-linked genes, there are at present no appropriate marker genes available for testing this possibility. In fact, it has been theoretically shown that appreciable amounts of linkage disequilibrium are created even between selectively neutral genes and ordinary dominant genes (23), if they are very closely linked and the population size is not large. Some permanent linkage disequilibria might arise between isozyme genes and gene blocks (such as inversions, as shown in the present studies).

In conclusion, it should especially be noted that although we must accept the importance of selection for some isozyme polymorphisms, it is becoming more evident that strong selection cannot account for the bulk of isozyme polymorphisms (25) [estimated amount of heterozygosity is about 12% (1)].

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