CLOSE LINKAGE OF EYE COLOR GENES IN *TRIBOLIUM CASTANEUM*¹

A. A. DEWEES AND A. E. BELL

Population Genetics Institute, Purdue University, Lafayette, Indiana 47907

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IN the course of linkage studies involving several new mutations affecting eye pigmentation in the flour beetle, *Tribolium castaneum*, it became apparent that while being nonallelic to each other and to previously reported eye mutants they were not randomly distributed in the genome. In fact, of the nine eye mutant loci thus far reported, six occur as closely linked members of three gene pairs. It is the purpose of this report to present these new linkage relationships in *T. castaneum* and to discuss the nonrandom distribution of these genes in terms of their apparent locational-functional relationships.

MATERIALS AND METHODS

Six nonallelic eye color mutations or gene loci in *Tribolium castaneum* were involved in the present study. A brief description along with their original citation follows:

1. pearl, *p*—autosomal recessive discovered in the Chicago Wild Type Stock (PARK 1937). Pupal and adult eyes are devoid of the normal black pigment and thus have a crystalline or pearl-white appearance. Black pigment in the ocular diaphragm causes the peripheral ommatidia to appear black (WOLSKY and ZAMORA 1960). A second recessive allele, pink, *p^{pk}*, discovered in a separate laboratory but from the same Chicago Wild stock, was used in certain crosses. It is similar to pearl, but a pinkish tinge develops in the ommatidia of older beetles. (LASLEY 1960).

2. ivory, *i*—autosomal recessive found in a population derived from Purdue Wild Type Foundation Population (called "A" in earlier reports). Eye is creamy white and resembles the pearl phenotype. Ivory is nonallelic to pearl, but is closely linked (BARTLETT 1962; BARTLETT and BELL 1966).

3. ring, *rg*—sex-linked recessive found in the Purdue Wild Type Foundation Population. Eye color is very similar to the ivory and pearl phenotypes in that the light or white center of the eye is circled by a darker marginal ring. (YAMADA 1962).

4. rose, *rs*—sex-linked recessive derived from the Purdue Wild Type Foundation Population. Not allelic to ring or red (LASLEY 1960), but resembles the red phenotype in that the central portion of the normally black compound eye has a reddish-pink color. The rose phenotype in old adults darkens toward wild type. (REYNOLDS 1964).

5. maroon, *m*—autosomal recessive discovered in an irradiated subpopulation of the Purdue Wild Type Foundation. Eye color of pupae and young adults is maroon or dark red. The pigmentation darkens with age so that by 10 days after eclosion the maroon phenotype overlaps wild type. (EDDLEMAN 1962; EDDLEMAN and HUDSON 1965).

6. ruby, *rb*—autosomal recessive, nonallelic to maroon, but in the same linkage group. Bicolored eye has reddish-brown central portion which darkens slightly with age but remains distinguishable from wild type. Discovered in a wild population collected from a feed storage bin at Southern Illinois University, Carbondale, Illinois (DEWEES 1963, 1965).

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All six gene mutations have complete penetrance and good expressivity but there is sufficient overlap in the expression of each linked pair to limit recombination tests to the repulsion or *trans* phase with the number of wild-type progeny assumed to represent one-half the total number of recombinants. Because no close outside markers are available for these three pairs of loci, all testcross progeny classified as recombinant wild type were individually progeny-tested to determine their exact genotypes.

Since the numbers of recombinants observed during these experiments were small compared with the total progeny sampled, use of the normal approximation to the binomial for setting up confidence intervals and making tests of significance is not valid. Tabulated values of fiducial limits for the binomial distribution presented by STEVENS (1942) were used to set up 95% fiducial limits for the recombination fractions.

Reciprocal matings were made in all linkage tests of autosomal genes with age of parents ranging from 8 to 15 days. Single-pair matings in $\frac{3}{4}$ oz creamers (20 ml glass bottle with cardboard pull-cap) were used for all crosses except where indicated. In certain crosses demanding large numbers of offspring, mass matings in large plastic boxes (12 cm $\times$ 17 cm $\times$ 6 cm) were made. With the exception of the ruby-maroon tests, progeny were classified one to two weeks after adult emergence. Since maroon beetles darken with age to resemble the wild-type black color, progeny from these tests were checked as late pupae or young adults. In all matings the beetles were cultured in standard medium (95% whole wheat flour and 5% dried brewer's yeast) in an environmental control chamber maintained at 33°C and 70% relative humidity.

RESULTS

Preliminary test matings revealed that the six eye color mutations involved here represented three pairs of closely linked gene loci. The members of each pair were identified as nonallelic since wild-type phenotype was observed for both the *cis* and *trans* double heterozygotes. Also, the double homozygote for each gene pair resembles the single homozygote having the lightest eye color, i.e., *rg rs/rg rs* has the ring phenotype, *rb m/rb m* has the ruby phenotype and *p^{pk} i/p^{pk} i* has the lighter ivory phenotype. Summaries of the recombination data will be presented beginning with the least closely linked loci.

ring-rose pair: As indicated in Table 1, the recombination fraction between *rg* and *rs* calculated by pooling the data from the three crosses is .032. Mating Groups B and C of Table 1 included the sex-linked pygmy (*py*) locus for three-point linkage. *rose* was located between ring and pygmy $18.15 \pm .72$ map units to the left of pygmy. SOKOLOFF *et al.* (1966) have described the platinum eye locus (*pte*) as sex-linked and located about 18 units to the left of pygmy. Subsequent allelism tests revealed that *pte* (kindly supplied by A. SOKOLOFF) is allelic to *rg*.

ruby-maroon pair: As can be seen in Table 2, the recombination rate in the

TABLE 1
Recombination between ring and rose

Mating	Single-pair matings	Phenotype of male progeny			Recombination fraction	95% fiducial limits
		Mutant	Wild	Total		
A. <i>rs</i> +/+ <i>rg</i> ♀ $\times$ + + ♂	49	997	16	1013	.0316	(.0182, .0510)
B. <i>rs</i> +/+ <i>rg</i> ♀ $\times$ + + ♂	50	836	10	846	.0236	(.0114, .0433)
C. <i>rs</i> +/+ <i>rg</i> ♀ $\times$ + + ♂	50	1976	36	2012	.0358	(.0252, .0949)
Totals	149	3809	62	3871	.032	(.0246, .0410)

male parents is about 12 times greater than that observed in the female parent: .0148 versus .0012. These two rates differ statistically at the 5% probability level. One mating from a total of 97 fertile matings was excluded from the B group of Table 2. It had produced five wild-type progeny among a total of 120, but all five died before producing progeny in test matings and their genotypes could not be established. SOKOLOFF (1964) also reported crossing over in the male to be significantly greater than in the female for several loci in linkage group VII of *T. castaneum*. JOHNSON (1966) examined this problem further utilizing loci of linkage groups IV and VII and proposed that differences in male and female recombination rates are obtainable when the two sexes differ in the distribution of a single chiasma.

The parental crosses for this gene pair were actually $m + +/+ rb j \times m rb + /m rb +$ with j representing the recessive body color gene jet located 26 map units from the maroon locus (EDDLEMAN and HUDSON 1965). From this cross it is possible to obtain the recombination rate between rb and m and also their position in relation to jet. A regular three-point linkage test was not performed because of the difficulty in distinguishing between ruby and maroon and also because of the added time needed to check the progeny, first as pupae for eye color and later as adults for body color. The sequence of these three genes was determined by progeny testing 34 of the wild-type recombinants of Table 2 in mating to the $+ + j/+ + j$ stock. Knowing that the map distance between m and j is 26 recombination units, one would expect 26% or less (interference would cause less) of the wild-type recombinants to contain the j allele if the gene sequence were $m-rb-j$. On the other hand, a gene sequence of $rb-m-j$ would cause this expectation to increase to 74% or more. In fact, only two of the 34 or 6% of the wild-type recombinants possessed the j allele, indicating that the gene sequence is $m-rb-j$.

pearl-ivory pair: These two loci represent the closest linkage thus far reported in *T. castaneum*. Obtaining a recombinant-type chromosome containing both p^{pk} and i genes by individually test mating F_2 mutant males (from $p^{pk} + /+ i \times p^{pk} + /+ i$ matings) to both pink and ivory females is laborious but was attempted before the closeness of the linkage was realized. This approach was abandoned

TABLE 2
Recombination between ruby and maroon

Mating	Single-pair matings	Phenotype of progeny			Recombination fraction	95% fiducial limits
		Mutant	Wild	Total		
A. $rb m/rb m \text{ } \text{f} \times rb +/+ m \text{ } \text{m}$	87 I*	3986	35	4021	.0174	(.0121, .0242)
	II	4039	25	4064	.0123	(.0080, .0182)
	I and II combined	8025	60	8085	.0148	(.0113, .0191)
B. $rb +/+ m \text{ } \text{f} \times rb m/rb m \text{ } \text{m}$	96 I	6414	7	6421	.0022	(.0009, .0045)
	II	6824	1	6825	.0003	(.00001, .0017)
	I and II combined	13238	8	13246	.0012	(.0005, .0024)

* I and II represent consecutive four-day egg collections.

after 500 mutant F_2 males were proven to be either $p^{pk} +/p^{pk} +$ or $+i/+i$ genotype.

While the forward mutation rates for the pearl and ivory loci in germinal tissues of *Tribolium castaneum* have not been studied, the mutation approach for obtaining a double homozygous tester stock was undertaken. This involved a search for the mutant phenotype (pearl-like eye color) among the normally occurring wild-type F_1 offspring from a cross of pink to ivory. Twenty $p^{pk} +/p^{pk} + \times +i/+i$ matings were made and the progeny were checked for mutant eyes. Among 2292 F_1 offspring observed, one had mutant eyes similar in color to ivory. It was determined that the mutation occurred on the $p^{pk} +$ chromosome, presumably from $+$ to i . A tester stock was then established with the genetic properties of the double homozygote $p^{pk} i/p^{pk} i$ and the phenotype of ivory.

From Table 3 it can be seen that no wild-type recombinants were observed among more than 10,000 progeny from test mating the double heterozygous male (Mating A). However, crossing over in the female at a rate of .00062 was observed with two of the four recombinant types testing as $p^{pk} i/+ +$; the other two died before being tested. If it can be assumed that the recombination rates in males and females are equal for these two loci and that the observed difference is due to sampling error, then a combined estimate of the recombination rate between the pearl and ivory loci is .000345.

Since no wild-type recombinants were produced by crossing over between p^{pk} and i in the male parent, this area was re-examined by determining the recombination rate between the p (pearl) allele and i . Mass matings were used in these crosses to facilitate the collection of a large number of progeny. Here again, if the rate of crossing over between pearl and ivory is equal in the two sexes, a combined estimate of the recombination fraction of .0003 is obtained (Table 4). The hypothesis that the rates are equal in the two sexes was accepted at the 5% level by use of Table 14 in GOLDSTEIN (1964). Four of the five recombinant types were tested and revealed the recombinant genotype, $p i/+ +$.

Since the few wild-type recombinants could possibly result from back mutation at either the p or i locus in the heterozygous parent, the magnitude of these mutation rates was investigated. No back mutations were observed among 10,800,

TABLE 3
Recombination between pink and ivory

Mating	Single-pair matings	Phenotype of progeny			Recombination fraction	95% fiducial limits
		Mutant	Wild	Total		
A. $p^{pk} i/p^{pk} i \text{ } \varnothing \times p^{pk} +/+i \text{ } \delta$	109 I*	4977	0	4977		(0, .0012)
	107 II	5372	0	5372		(0, .0011)
	I and II combined	10449	0	10449		(0, .0006)
B. $p^{pk} +/+i \text{ } \varnothing \times p^{pk} i/p^{pk} i \text{ } \delta$	107 I	6118	2	6120	.00065	(.00008, .00236)
	109 II	6710	2	6712	.00060	(.00007, .00215)
	I and II combined	12828	4	12832	.00062	(.00017, .00159)

* I and II represent consecutive four-day egg collections.

TABLE 4

Recombination between pearl and ivory

Mass mating	Phenotype of progeny			Recombination fraction	95% fiducial limits
	Mutant	Wild	Total		
A. 200 $p^{pk} i/p^{pk} i$ ♀ ♀ × 159 $p +/+ i$ ♂ ♂	15123	3	15126	.00040	(.00008, .00116)
B. 196 $p +/+ i$ ♀ ♀ × 185 $p^{pk} i/p^{pk} i$ ♂ ♂	20168	2	20170	.00020	(.00002, .00072)
A and B combined	35291	5	35296	.00028	(.00009, .00066)

18,000 and 10,000 progeny from the i , p and p^{pk} stocks respectively. Therefore, recombination rather than mutation probably accounts for the recombinant genotypes.

Pseudoallelism has not been reported for *Tribolium* probably because tests with the resolving power required to reveal such cases have not been undertaken. In view of the close linkage between pearl and ivory it was decided to test the p and p^{pk} alleles of the pearl locus for pseudoallelism. From reciprocal crosses of $p +/p^{pk} i \times p^{pk} +/p^{pk} +$ in mass matings, all 36,654 progeny were mutant, indicating that at this level of resolution no recombination occurs between these two alleles.

DISCUSSION

Nine gene loci affecting eye pigmentation have now been mapped for *Tribolium castaneum*. The nonrandom grouping of these genes is clearly evident from Figure 1, where seven of the nine loci are shown in relation to other marker genes. The two remaining known eye color loci, white (EDDLEMAN and BELL

FIGURE 1.—Close linkage at three pairs of gene loci affecting eye pigmentation shown in relation to other known markers. (The sequence of ivory and pearl in relation to pegleg has not been determined.)

1963) and chestnut (EDDLEMAN 1961) are located in linkage groups IV and VII, respectively. The significance of this gene arrangement might best be explained in terms of the probable mechanism by which these genes affect eye color.

Some gene mutations may cause structural changes in the pigment granules thereby resulting in eye color changes as has been reported for *Drosophila* (NOLTE 1961). Others probably act by blocking steps in the pigment synthesis pathway through the production of either an inactive enzyme or no enzyme at all. If the accumulation of the precursors has no visible effect, those genes that block single steps in the same pathway often produce very similar phenotypes. For example the brown eye pigment system (ommochromes) in *D. melanogaster* is blocked at various steps by four mutant genes; vermilion, cinnabar, scarlet and cardinal, all producing nearly identical phenotypes (ZIEGLER 1961). Mutant eye phenotypes which result from blocks in this same pathway have also been reported for such insects as the horsefly, blowfly, honey bee, silkworm and the cockroach (WAGNER and MITCHELL 1964). The rosy and maroon-like eye mutants in *Drosophila* are both deficient in the enzyme xanthine dehydrogenase, which is presumably important in the red pigment system, and both produce similar phenotypes (GLASSMAN and MITCHELL 1959). SOKOLOFF (1966) suggests that in the red eye color mutant of *T. castaneum* the red pigment is probably an ommochrome, however there have been no reports of studies on the chemistry of pigment synthesis in this species.

It is not uncommon to find functional-locational relationships of genes in micro-organisms. For example, the nine genes which control the steps in the synthesis of histidine in *E. coli* are linked together as adjacent structural genes of the histidine operon (AMES and HARTMAN 1963). According to the operon hypothesis of JACOB and MONOD (1961), the adjacent structural genes provide a code for enzymes which catalyse steps in the same metabolic pathway. While functionally related genes are clustered in one organism, they appear to be more randomly distributed in others. For example, the genes controlling the steps in the pathways of leucine, tryptophan and histidine synthesis are grouped into operons in bacteria while in *Neurospora* they are, for the most part, scattered among the chromosomes (HOROWITZ 1965). In reviewing material on the evolution of biosynthetic pathways, HOROWITZ (1965) proposes that the operon or gene cluster arrangement of bacteria is more primitive than the *Neurospora* gene arrangement and relates this to the possibility that the evolution of chromosomes brought with it a more elaborate genetic control system which eliminated the need for the operon mechanism. One might expect to find, then, in higher organisms a scattering of functionally related genes with possible vestiges of a primitive gene system remaining in the form of closely linked genes affecting the same character.

If it can be assumed that the synthesis of eye pigment in *T. castaneum* is a multi-step process, with the eye mutants representing blocks at various steps in this pathway, then the instances of close gene linkage reported here can be thought of as vestiges of a more primitive gene system in which there has been an incomplete breakup during chromosomal evolution.

Present day knowledge of gene evolution in higher organisms other than Dro-

sophila is sufficiently primitive that other hypotheses on the origin of closely linked loci should be considered. For example, the process of tandom duplication of genes with gradual functional differentiation has been proposed by LEWIS (1945) as an explanation for closely linked, functionally related genes. LEWIS (1967) views this process as a possible mechanism for the buildup in higher organisms of gene complexes associated with biosynthetic pathways of more recent origin. The nonallelic relationships and the magnitude of the recombination values for the three gene pairs reported here do not support the type of gene complex that LEWIS describes; however, the process of tandom duplication with functional differentiation remains a possible explanation for the nonrandom distribution of eye color loci in *T. castaneum*.

An even less likely possibility, in the authors' opinion, causing the close linkage would be chromosomal aberrations associated with one or both loci involved in each of the three closely linked pairs. Considerable evidence argues against this possibility. Only one of the six mutants, maroon, was discovered in irradiated material. All mutants are recessive and show normal fertility. Independently occurring alleles have been reported for the pearl, maroon, and ring loci to support the point mutation concept as opposed to the simultaneous occurrence of a chromosomal aberration with each new eye mutation. Furthermore, all three closely linked pairs have at least one outside marker which maps approximately the same distance from each locus within a pair. None of the above is characteristic of major chromosomal aberrations. While minor aberrations might go undetected, they would not refute the nonrandom distribution of these gene loci.

Further studies of pigment metabolism in reference to the eye mutants of *Tribolium* should aid in the interpretation and understanding of these cases of close linkage.

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SUMMARY

Of the nine reported gene loci affecting eye color in *Tribolium castaneum*, close linkage has been found for three different pairs: ring and rose (sex-linked; 3.2% recombination); ruby and maroon (autosomal; 1.48% in males, 0.12% in females); ivory and pearl (autosomal; .03%). This is clearly not a random distribution and may represent a breakup of a primitive gene system.

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