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The chromosomal location of the malate dehydrogenase and the phosphoglucomutase loci in *Chironomus* and their relationship with a sex determining region

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ABSTRACT

The location of the phosphoglucomutase (Pgm) locus in several Australian Chironomus species has been found to be near the centromere of the CD chromosome, as reported in a European species, possibly on arm D. The malate dehydrogenase (Mdh -1) locus of the Australian species is shown to be in the same region. The use of the holarctic species, Ch. tentans , in which arms C and D are separated in the karyotype, indicated that the Mdh locus is actually on arm C. The Pgm locus could not be more accurately located using Ch. tentans as it was invariant in our stocks, although the locus appears to have been duplicated.

The relationship of these two loci to the known CD sex determiner is useful in studies of the variability in the position of the sex determiner within this genus and examples of the appropriate analyses are provided.

INTRODUCTION

In our recent studies on sex determination of *Chironomus* species (Martin and Lee 1984a), we have become interested in the use of genetic markers to more accurately map the location of the male determining (MD) genes. However, the formal genetics of the genus *Chironomus* has been relatively little developed, largely because visible markers have rarely been recorded. A partially sex linked eye colour mutant, recorded in *Ch. pallidivittatus*, was ascribed to the CF chromosome which carries the MD in this species (Beermann 1955). The situation with respect to protein and enzyme loci is a little better. Tichy (1968, 1970, 1975) and Thompson et al (1969) investigated the haemoglobins of *Ch. tentans* and *Ch. pallidivittatus* and located the genes concerned. These were mostly on arm D but two of the genes were on the associated arm E. A gene which regulates the arm E loci has also been located on this arm (Thompson and Horning 1973). Rothen (1978) studied five enzyme loci in *Ch. nuditarsis*, mapping some of them to particular chromosomes. The 5S RNA locus has been identified in both *Ch. tentans* (Wieslander et al 1975) and *Ch. thummi* (Bäumlein and Wobus 1976). In the case of *Ch. thummi*, the locus was identified as region B3c-e of chromosome 2R (corresponding to arm C in the scheme of Keyl, 1961), while in *Ch. tentans* the locus was at a quite different location, despite the rather similar designation of this region as 2A of chromosome 2. This arm corresponds to arm B in the Keyl scheme. Recently we have been able to locate the 5S RNA locus in one of the Australian species, designated *Ch. alternans* c (see Martin and Lee 1981). In this species the site is at about 3A5-6 or arm B (Martin, Webb and Lee, unpubl. data), corresponding more with the location reported for *Ch. tentans*. The only other markers localized have been the rRNA and Balbiani ring genes of a number of species, but these are cytologically recognizable, except for the temperature-induced T-BRIII of *Ch. thummi* (Carmona et al 1985).

The phosphoglucomutase (*Pgm*) locus was of immediate interest to us because Rothen (1978) had indicated that it was near the centromere of the CD chromosome in *Ch. nuditarsis*. Also our preliminary results indicated that a malate dehydrogenase locus (*Mdh* -1) was in a similar location in some Australian species. If the location of these two loci could be confirmed in other species, they would be of condsiderable value for mapping MD genes, since a location near the CD centromere has been indicated for several species (Martin et al 1980, Martin and Lee 1984a). A major problem was determining the location of these enzyme loci

in relation to the centromere. In the absence of any other markers, inversion polymorphisms were the only means of mapping these genes. As indicated by Rothen (1978) and by the results below, this is not sufficiently accurate to indicate whether the loci actually lie on the arm C or the arm D side of the centromere. The question could potentially be answered by using *Ch. tentans*, where arms C and D are physically separated and occur on different metacentric chromosomes, with alternative banding sequences against which the inheritance of the enzyme loci could be compared.

MATERIALS AND METHODS

The material used comprised the various forms of *Chironomus oppositus* and the closely related *Ch. alternans* c (Martin and Lee 1984a), *Ch. duplex*, the New Zealand species *Ch.* sp.a (Martin and Lee 1984a) as well as a European and a North American stock of *Ch. tentans*. The European stock came from Plön, Germany (sent by Dr. H.Tichy, Tübingen), while the North American stock, from Winnipeg, Manitoba (sent by Dr. B.Townsend, Winnipeg), belongs to the East Canadian race (Acton and Scudder 1971). Rearing and breeding techniques, and the protocol for analysis using hybridization have been detailed by Martin et al (1980). Late fourth instar larvae were sexed and prepared for both cytological and electrophoretic analysis. The head and thorax of each larva was removed into a numbered vial of cytological fixative (3 parts absolute ethanol: 1 part glacial acetic acid) for subsequent preparation of salivary chromosome squashes by the usual technique (Porter and Martin 1977). The remainder of the larval body was placed into an identically numbered well of a plastic microtitre plate. Each well contained 15 µl of 0.1M Tris-EDTA-Borate, pH 8.1 (Shaw and Prasad 1970). The plates were stored at -60°C until required for electrophoresis.

Electrophoresis was performed in a horizontal bath at 5°C on 10 x 17 cm Cellogel sheets (Chemerton). These sheets were presoaked in the gel buffer for at least 30 min before being set up in the bath with the electrode buffer. The buffers used for the two systems were:

a. Phosphoglucomutase (Pgm) - The gel buffer was 0.1M Tris-EDTA-Borate, pH 8.1, the electrode buffer 0.1M Borate (Shaw and Prasad 1970). The gels were run for 2 1/4 hr at 250V, constant voltage. The staining mixture was: 20ml 0.1M Triethanolamine hydrochloride, pH 7.6 (McKenzie and Dawson 1969); 85 mg Glucose-1-phosphate; 100 mg $MgCl_2.6H_2O$; 25 µl Glucose-6-phosphate dehydrogenase (NAD-dependent); 10 mg ıNAD; 8 mg PMS; 10 mg MTT. In early runs the NADP dependent G-6-PD was used, but this was more expensive in that it required 10 mg of NADP instead of the NAD (Buth and Murphy 1980).

b. Malate dehydrogenase (Mdh) - Initial runs were made using a one in ten dilution of the 0.1M phosphate - citrate buffer, pH5.8, of McKenzie and Dawson (1969) for both the gel and electrode buffers. While this was suitable for the larger species, it proved unsuitable for the smaller species such as *Ch. oppositus*. Later runs therefore employed a one in eight dilution of 0.1M Citric acid - Sodium citrate, pH 5.8 (McKenzie and Dawson 1969), which gave good results with all species. Gels were run for 2 1/2 hr at 250V, constant voltage. The staining mixture was: 10 ml 0.05M Tris-HCl buffer, pH 8.6; 30 mg DL-Malic acid; 20 mg ıNAD; 10 mg PMS; 10 mg MTT.

The results were tested using Chi-square tests, employing Yates correction in the 2x2 contingency analyses. The significance level of the Chi-square results in the text is indicated as in the Tables.

RESULTS AND DISCUSSION

Terminology: Electromorph bands have been numbered in order of migration rate, the fastest moving band being numbered "1". However the homology of bands between species could not always be determined since individuals with the appropriate genotype were not available in the laboratory at the same time. Therefore numbers alone were used for members of the *Chironomus oppositus* group, following Martin and Lee (1987), while a letter prefix was used for the other species: "a" for *Ch.* sp.a, "d" for *Ch. duplex*, and "t" for *Ch. tentans*. Possible homologies between the bands of the various species are indicated below.

Pgm

The results obtained were similar to those of Rothen (1978) and Hilburn (1980), in that homozygotes showed only a single band and heterozygotes two bands. The number of electromorphs present and the relative mobilities varied between the species. Nine bands were found in members of the *Ch. oppositus* group, of which band 6 was the most common, with bands 4, 5 and 7 occurring at relatively low frequencies. The other bands were rare. Two bands were present in the material of *Ch.* sp.a, a^1, which appears equivalent to band 3 of *Ch. oppositus*, and a^2, which appears equivalent to band 5. The a^2 electromorph was more common in the ten egg masses from which the laboratory stocks were produced. Two bands were also present in *Ch. duplex*, d^1, which appears equivalent to band 5 and a^2, and the more common d^2, which appears equivalent to band 6.

All individuals examined of *Ch. tentans*, regardless of origin, showed two bands, apparently equivalent to bands 3 and 5. Since this pattern was invariant even in the offspring of the sibs of tested individuals, it was concluded that the locus was probably duplicated in this species. In one experiment duplicate strips were stained at room temperature and at 42°C. At the higher temperature staining was much paler and could not be detected in

five of the eleven individuals tested. In the other five larvae, the fastest migrating band was inactivated and only the slower band was stained. This is compatible with there being two loci, one of which is more sensitive to higher temperature than the other. However the absence of any variation at either locus rendered the species unsuitable for our purpose in trying to map the locus and no further experiments were carried out with *Ch. tentans*.

Hybridizations between *Ch. duplex* and *Ch.* sp.a: Since these two species have different common alleles and can be hybridized under laboratory conditions, it is possible to gain at least some information on the chromosomal location of the *Pgm* locus from an analysis of backcrosses. The F1 hybrids showed a band corresponding to to those of each parent (a^2 and d^2 in the specimens examined). This suggests that the electromorphs are indeed alleles at a single locus. These hybrids had a very low fertility, possibly as the result of failure of bivalent formation in meiosis. This is to be expected if meiotic pairing reflects the pairing observed in the polytene chromosomes, where the homologues were virtually unpaired. No successful backcrosses were obtained from hybrid males, but the mating of a male of *Ch.* sp.a with a hybrid female produced a number of egg masses with some fertile eggs, although less than 10% showed any development. Mature larvae were obtained from four egg masses. However, because of the small number of larvae from each egg mass, the results were pooled as this should not affect the interpretation of the data. Also, since we are only interested in the parent from which each allele arose, rather than which particular allele was present, the results in Table 1a show the alleles only as "a" (although both a^1 and a^2 were present), or "d" (which were all d^2). The results clearly indicate that the locus is not on chromosome I or chromosome III. The majority of the data are in agreement with the locus being on chromosome II (the CD chromosome) as previously reported for *Ch. nuditarsis* (Rothen 1978). There are two exceptions, larvae which were homozygous for chromosome II from *Ch.* sp.a, but which carried an allele from each species. These larvae were the only two males from one of the egg masses, but whether this is relevant is uncertain since no satisfactory explanation of these individuals can be advanced. If the locus is near the centromere as suggested by other data (Rothen 1978 and see below), a double cross-over event would be required within a distance which did not lead to visibly disturbed pairing in the polytene chromosomes. This seems unlikely, particularly since no results attributable to single cross-overs were observed. In the absence of any confirmed recombination, the results give no additional guide to the position of the locus on chromosome II.

Ch. oppositus: None of the hybridization and backcross experiments conducted with the various forms of *Ch. oppositus* or with *Ch. alternans* (Martin et al 1980, Martin and Lee 1984a) produced any information relevant to the location of the *Pgm* locus, since all the stocks used were homozygous for band 6. Even data from wild collected or laboratory bred egg masses was difficult to obtain because of the low frequency of simultaneous polymorphism for inversion sequences on the CD chromosome

Table 1. Inheritance of the electromorphs of the *Pgm* & *Mdh* loci in relation to the sequences of the three chromosomes in the backcross of a hybrid (*Ch.* sp. a/*Ch. duplex*) female to a *Ch.* sp. a male.

		Chrom I		Chrom II		Chrom III	
a. *Pgm*		a/a	a/d	a/a	a/d	a/a	a/d
	a/a	7	4	11	-	6	5
	a/d	6	5[a]	2	9	5	6[a]
	Total	13	9	13	9	11	11
		$X^2_1 = 0$		$X^2_1 = 12.03^{***}$	$X^2_1 = 0$		
b. *Mdh*							
	a/a	8	4	12		7	5
	a/d	3	3[a]	-	6[a]	4	2[a]
	Total	11	7	12	6	11	7
		$X^2_1 = 0.03$		$X^2_1 = 13.78^{***}$		$X^2_1 = 0.03$	

[a] Includes one larva which was triploid, at least for most of the genome and apparently resulted from a largely unreduced egg.

a = allele or chromosome derived from *Ch.* sp. a
d = allele or chromosome derived from *Ch. duplex*

* p<.05 ** p<.01 *** p<.001 (also in Tables 2-6)

Table 2. Associations of *Pgm* alleles with arm D sequence in *Ch. oppositus* f. *whitei* egg masses (data of Kuvangkadilok 1983).

Egg mass	Sex	Pgm genotypes	Arm D sequence				Total
Albert Park, Vic.			D_1/D_1	D_1/D_4			
Egg mass # 3	Female	4/4	-	11			11
	Male	4/5	22	-			22
			$X^2_1 = 28.65^{***}$				
Mitcham, Vic.			D_1/D_1	D_1D_3			
Egg mass # 2	Female	4/4	65	-			65
	Male	4/4	1	-			1
		4/5	-	47			47
			$X^2_1 = 104.98^{***}$				
			D_1/D_1	D_1D_4			
F_1 egg mass # 1	Female	4/4	1	-			1
		4/5	-	60			60
	Male	4/4	52	-			52
		4/5	-	1			1
			$X^2_1 = 102.26^{***}$				
F_1 egg mass # 2	Female	4/4	123	-			123
	Male	4/5	-	57			57
			$X^2_1 = 175.41^{***}$				
			D_1/D_1	D_1/D_3			
Egg mass # 7	Female	4/4	50	-			50
		4/5	-	63			63
	Male	4/4	46	-			46
		4/5	-	33			33
			$X^2_3 = 3.10$				
North Box Hill and Mitcham, Vic.			D_1/D_1	D_1/D_2	D_1/D_3	D_2/D_3	
F_1 egg mass # 2	Female	4/4	20	28	-	-	48
	Male	4/5	-	-	10	32	42
			$X^2_3 = 86.03^{***}$				
			D_1/D_1		D_1/D_4		
F_1 egg mass # 3	Female	4/4	111		-		111
	Male	4/5	-		98		98
			$X^2_1 = 205.00^{***}$				

Small classes were combined into the appropriate larger groups.

and the *Pgm* locus. A wild collected egg mass (#22) of form *oppositus* from Moggs Creek, Victoria was informative. In this form the sex determiner is known to be on the CD chromosome near the centromere (Martin and Lee 1984a). The 19 larvae reared from the egg mass were either D1/D1 (11) or D1/D2 (8, of which 1 was not tested for Pgm), but with no difference between the sexes. Therefore it can be concluded that the female parent was heterozygous D1/D2 and the male parent homozygous D1/D1. The Pgm genotypes of the larvae were 3/4 (7) or 4/5 (11), with all 3/4 individuals being D1/D2 and all 4/5 individuals being D1/D1 ($X^2_1 = 14.04^{**}$). There was no difference between the sexes for Pgm genotype ($X^2_1 = 0.44$). This is consistent with the female parent also being heterozygous for Pgm alleles 3/5, allele 3 being on the chromosome carrying D2 and allele 5 being on the chromosome carrying D1. The male parent would have been homozygous for Pgm alleles 4/4.

These data support the previously unpublished findings of Kuvangkadilok (1983). On the basis of her results, reproduced in Table 2, she concluded that the *Pgm* locus was closer to the proximal breakpoints of the arm D inversions than it was to the MD, probably lying between region 9B4 and the site of the MD. Her results suggest that about 0.4% recombination occurs between the *Pgm* locus and the MD.

The knowledge of the linkage between the *Pgm* locus and the CD sex determiner allows us to utilize other egg mass data from

Table 3. Association of *Pgm* alleles with sex in egg masses of species with a sex determiner on the CD chromosome.

Species	Sex	Pgm Genotype				Total	X^2	df
Ch. oppositus :								
form *whitei*		3/4		4/4				
Aireys Inlet, Vic.	Female	6		-		6	8.33**	1
	Male	-		6		6		
		2/4		4/4				
Moggs Creek, Vic.	Female	6		-		6	5.49*	1
	Male	1		5		6		
form *oppositus*		2/3	2/4	3/4	4/4			
Moggs Creek, Vic.		1	6	3	4	10	13.14**	3
		-	-	4	-	10		
Ch. alternans b		2/3	2/3	3/3				
North Balwyn, Vic.	Female	-	5	4		9	7.09*	2
	Male	3	6	-		9		

species in which the MD is known to be on the CD chromosome: *Ch.oppositus* forms *whitei* and *oppositus* , and *Ch. alternans* b (Martin and Lee 1984a). In these egg masses (Table 3), there was no inversion polymorphism on the CD chromosome but the inheritance of the Pgm alleles is clearly sex linked. This is most useful in the case of the egg masses of form *whitei* , since the location of the MD is polymorphic in this form (Martin and Lee 1984a) and the sex linked inheritance of the *Pgm* locus enables the MD to be ascribed to the CD chromosome in these two egg masses. The linkage of the *Pgm* locus to the CD chromosome was used to answer a different question in another egg mass from Moggs Creek, Victoria (#71). The larvae from this egg mass were found to be segregating inversions in four chromosome arms: with sequences D1 and D4, C1 and C3, E1 and E2, and A1 and A2. Sequences D4, C3, E1 and A1 were only present in heterozygotes. These sequences occur as polymorphisms in *Ch. oppositus* form *oppositus* , but they also occur in form *tyleri* (Martin and Lee 1984a). The egg mass could therefore be form *oppositus* or a hybrid backcross involving forms *oppositus* and *tyleri*. The data (Table 4) show that there is no evidence of sex linkage between the arm D sequences, the arm C sequences, or the Pgm alleles. This could simply mean that the female parent was heterozygous for both chromosome arms and the *Pgm* locus. However if this was the case there should be linkage between the arm D sequences and the Pgm alleles. As seen from Table 4, this is not the case. It must therefore be assumed that there is no MD on the CD chromosome. The egg mass is therefore unlikely to be form *oppositus* , but is most likely to be the result of the backcross of a male hybrid between forms *oppositus* and *tyleri* to a female of form *oppositus*. The heterozygosity in four chromosome arms would also support this conclusion. It is assumed that the MD is on arm G, coming originally from a form *tyleri* male (Martin and Lee 1984a). Unfortunately there are no known markers on arm G to confirm this hypothesis. The indication that natural hybridization is occurring has important implications for the possible origin of rare inversion polymorphisms, such as F2 and

Table 4. Segregation of the sequences of arms C and D, of the alleles of the *Pgm* locus and sex of the larvae of egg mass 71 from Moggs Creek, Victoria.

	Arm C		Arm D		Pgm		Total
Sex	C1/C1	C1/C3	D1/D1	D1/D4	3/4	4/4	
Female	7	5	6	6	3	9	12
Male	3	9	4	8	5	7	12
	$X^2_1 = 1.54$		$X^2_1 = 0.17$		$X^2_1 = 0.19$		
Pgm							
3/4	3	5	2	6			8
4/4	7	9	8	8			16
	$X^2_1 = 0.02$		$X^2_1 = 0.54$				

G2 (Martin and Lee 1984a), in form *oppositus*. This result suggests that the arm G sex determiner might also be present in some populations of form *oppositus* as a result of introgression.

Mdh

Again the results obtained were similar to those reported by Hilburn (1980) in that there were two loci present, with the more slowly migrating locus (*Mdh* -2) essentially monomorphic. Also in *Mdh* -1, the presumed heterozygotes showed three bands. Only the *Mdh* -1 locus will be considered further. The number of electromorphs present and the relative mobilities again varied between species, as with *Pgm*. However there were fewer electromorphs overall. Only four bands were found in members of the *Ch. oppositus* group, with band 3 the most common. The other three bands tended to be differentially distributed between the members of the group. Two bands were present in *Ch.* sp.a., a^1 which might be equivalent to band 1, and a^2 which appears equivalent to band 2. Band a^2 was by far the most common in the egg mass-derived sample. Two bands were also found in *Ch. duplex*, d^1 with a mobility between that of bands 2 and 3, and the more common d^2 which appears equivalent to band 3. Three bands were present in the samples of *Ch. tentans*. The most common band was t^2, which was present in both the Plön and Winnipeg samples and has a slightly slower mobility than band 3, while bands t^1, which appears equivalent to band 2, and t^3, which appears equivalent to band 4, were found only in the Winnipeg sample.

Hybridizations between *Ch. duplex* and *Ch.* sp.a: These crosses provided evidence that the electromorphs were alleles at a single locus, as well as giving some indication as to the chromosome on which the locus was situated. The F1 hybrids showed a three banded pattern indicative of heterozygosity for bands a^2 and d^2. The backcrosses were identical to those scored for the *Pgm* locus, and the data are presented in the same way in Table 1b. This data are clearly indicative that the locus is on chromosome II, the CD chromosome. Since no crossing over had occurred in chromosome II in the F1 females, the data give no indication of the relative position of the locus on this chromosome.

Ch. oppositus: As with the *Pgm* locus, the only relevant information here has come from wild collected egg masses, since the stocks used in hybridization crosses were all homozygous for band 3. Even the egg mass data are relatively sparse because of the low frequency with which segregation of both the enzyme locus and relevant inversions occurred in the same egg mass. Table 5 presents the results for the only three such egg masses found. In each case there is sex linked segregation of inversions in arm D, associated with segregation of the alleles of the *Mdh* locus. In this small sample there was no recombination between the Mdh alleles and either the MD or the arm D inversions. Larvae from one of the egg masses were also segregating sequences in arm B. As would be expected, there was no association between the sequences of this arm and either the MD or the *Mdh* locus.

Ch. tentans: Since the Winnipeg stock belongs to the East Canadian race, one of the arms of interest, arm C, should contain the sex determining loci (Martin and Lee 1984b). This was confirmed where a male from a backcross of a hybrid Winnipeg X Plön female to Winnipeg male (i.e. WWP) passed the North American derived sequence 1Lk to all male offspring and the European derived sequence IL to all female offspring (Table 6). The 1L sequence came originally from a female and so represents the 'X' chromosome in this series of crosses. The same cross also clearly indicates that the Mdh alleles are inherited in a sex linked manner (Table 6). The WWP male must have been heterozygous for alleles t^1 and t^2, the t^2 allele coming from the

Table 5. Segregation of the arm D sequences and the alleles of the *Mdh* locus in egg masses of *Ch. oppositus* f. *whitei*.

	Arm D			Total	Mdh		Total
Aireys Inlet, Vic.							
Egg mass 9	D_1/D_1	D_1/D_3	D_3/D_3		2/2	2/4	
Female	14	-	-	14	12	-	12
Male	-	14	-	14	-	12	12
		$X^2_1 = 24.14^{***}$			$X^2_1 = 20.17^{***}$		
Egg Mass 13					1/2	2/2	
Female	9	5	-	14	-	6	6
Male	-	6	4	10	6	-	6
		$X^2_2 = 13.09^{**}$			$X^2_1 = 8.33^{**}$		
West Preston, Vic.	D_1/D_1	D_1/D_4			2/2	2/4	
Female	8	-		8	-	9	9
Male	-	10		10	8	-	8
		$X^2_1 = 14.18^{***}$			$X^2_1 = 13.22^{***}$		

Table 6. Segregation of the sequence of arm C and the alleles of the *Mdh* locus of *Ch. tentans* in the cross of a WWP male (1L/1Lk; t^1/t^2) to a Winnipeg stock female (1Lk/1Lk; t^2/t^3).

	1Lk/1Lk; t^1/t^2	1Lk/1Lk; t^1/t^3	1L/1Lk; t^2/t^2	1L/1Lk; t^2/t^3	Total
Females	-	-	18	15	33
Males	11	9	-	-	20
		$X^2_3 = 52.97^{***}$			

Plön female and being on the homologue carrying sequence 1L, while the Winnipeg female must have been heterozygous for alleles t^2 and t^3. Sex linkage of the Mdh locus was clearly confirmed by three generations of self crosses derived from the original backcross. A total of 56 females scored from these three generations were all t^2/t^2, while the 55 males scored were all t^1/t^2 ($X^2_1=107.03^{***}$). This provides conclusive evidence that, in *Ch. tentans*, the Mdh locus is located on arm C.

CONCLUSIONS

The present data have confirmed that, as in the European species *Ch. nuditarsis* (Rothen 1978), the *Pgm* locus is located near the centromere of the CD chromosome, perhaps on the proximal region of arm D (Kuvangkadilok 1983). Our data indicate that the *Mdh* locus is also near the centromere of the CD chromosome, but probably on arm C assuming it occupies the same polytene band in the Australian species that it does in *Ch. tentans*. Both the enzyme loci are potentially useful for identifying the presence of the MD when it occurs near the CD centromere in some of the forms of *Ch. oppositus* or in other Australian species. If stocks segregating the two loci can be obtained it may be possible to more accurately map the MD in relation to these loci. The data presented for Moggs Creek egg mass #71 also indicate the use of these data in clarifying other problems relating to hybridization between the forms of *Ch. oppositus* and the resultant introgression of genetic material.

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