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Studies of the species barrier between *Drosophila madeirensis* and *Drosophila subobscura*

II. Genetic analysis of developmental incompatibilities in hybrids

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The genetic analysis of two abnormal characters, extra sex combs and abnormal head shape in hybrids between *Drosophila madeirensis* and *Drosophila subobscura*, revealed a major effect of the X chromosome in both cases. Autosomes also play a role in determining these abnormalities, the E chromosome in case of extra sex combs, the E and O chromosomes in case of abnormal head shape. Autosomes do not cause the two abnormal characters, without interacting with the X chromosome.

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One important way in which the process of speciation can be studied is by examining the hybrids of two closely related species. This can be very informative provided that at least one sex in one of the reciprocal crosses is viable, fertile, and able to produce progeny when mated to one, or both, of the parental species. The main core of this type of study, is concentrated on the analysis of hybrid male sterility factors in *Drosophila* (DOBZHANSKY 1936; COYNE 1984, 1985; VIGNEAULT and ZOUROS 1986; KHADEM and KRIMBAS, in press). Some analysis of female sterility has also been carried out (ORR 1987, 1989). However, in addition to the sterility factors that prevent the gene flow between two species, other characters such as viability and morphological or developmental abnormalities of the hybrids may also contribute to the genetic barrier between species, regardless of whether they are involved in the speciation process or are a byproduct of speciation. The contribution of these characters may be a partial rather than a complete genetic isolation. In order to have a better understanding of the number of factors (genes) determining such characters, their location and their possible interaction, we have analyzed two morphologically abnormal characters and their possible correlation with sterility/inviability in male hybrids between the closely related species of the *obscura* group, *D. subobscura* and *D. madeirensis*.

The hybrids between these two species show many different arrays of abnormalities. Some of these characters appear consistently in F_1 and backcross progenies, and are therefore suitable for genetic analysis. These include extra sex combs in the hybrid males and abnormal head shape. Other characters, however, appear either randomly or rarely, such as: abnormal number of wings, third leg changed to a wing like shape, extra hairs on the second longitudinal vein of the wing, merging of the two synthetic eyes and abnormal abdominal tergites.

Material and methods

Strains. — The following strains of *Drosophila subobscura* were used: 8 (Crete); ch cu (cherry eyes-curled wings); ma int (maroon eyes- interrupted wing); pp pl (poppy eyes-plexus wings) and nt (net wings). Two chromosomally monomorphic strains of *Drosophila madeirensis* were also used.

Markers. — In addition to the seven visible mutations mentioned above, three electrophoretic markers (Peptidase 3, Isocitric dehydrogenase, Malic enzyme), antenna colour (a species marker) and two chromosomal markers (inversion 16BCD and MAD

1) were used. Antenna colour is light in *D. madeirensis* and dark in *D. subobscura*. This marker is located on the X chromosome and shows Mendelian inheritance. Further details about strains and markers are given in KHADEM and KRIMBAS, in press.

Crosses. — Females of *D. madeirensis* were crossed to *D. subobscura* males; fertile F_1 female progeny were backcrossed to *D. subobscura* males (F_1 males are sterile). B_1 males were then analysed for two abnormal characters, extra sex combs and abnormal head. Each type of cross (using different strains) were performed 2 to 3 times and the homogeneous results were added up.

Occasionally, other types of crosses were also performed, they are explained in the text.

Results

Extra sex combs

Both *D. madeirensis* and *D. subobscura* males have two sex combs in the first and second tarsal segment of the first pair of legs. The teeth in *D. madeirensis* are more numerous (mean 31.5±0.5) than in *D. subobscura* (mean 20.7±0.18) (KRIMBAS and LOUKAS 1984). All F_1 hybrid males from the cross of *D. madeirensis* females to *D. subobscura* males have, in addition to the sex combs on the first pair of legs, sex combs in the second and third pairs of legs (extra sex combs, *esc*). This phenotype is easily distinguished in all F_1 males, although some variation does exist from individual to individual in the number of teeth and the pair of legs on which the character appears. F_1 females crossed to either of the parental species produced backcross males with the following phenotypes:

♀♀F_1 × ♂♂ *sub*		♀♀F_1 × ♂♂ *mad*	
B_1:	318♂♂ esc	B_1:	127♂♂ esc
	1757♂♂ normal		781♂♂ normal
	2075 total number of males		908 total number of males

$\chi^2 = 0.8$ for 1 df, not significant

In most of the cases, the F_1 males from the reciprocal cross (i.e., *D. subobscura* females crossed to *D. madeirensis* males) do not show the *esc* phenotype. Only a few individuals display it and to a lesser degree, with fewer teeth, which only exist on the second pair of legs. The number of individuals having *esc* in this case is 4 compared to 171 normal flies. This difference between the F_1 males of the two reciprocal crosses may indicate an influence of the sex chromosomes and/or the cytoplasm.

In a search for the influence of the X chromosome, we have determined the origin of the X chromosome by using antenna colour as a marker in 287 backcross males originated from F_1 females to *D. subobscura* males. The results are shown below:

	esc	normal
sub X chrom.	0	135
mad X chrom.	39	113

The Y chromosome is of *subobscura*. This suggests that only males with a *madeirensis* X display the extra sex comb character. This interpretation also holds for the F_1 males of the same cross, where the X is of *madeirensis*, Y of *subobscura*, and the autosomes are of mixed origin. The origin of the cytoplasm in all the cases is from *madeirensis*. The absence of the character in most of the F_1 male hybrids with an X of *subobscura* and a Y of *madeirensis*, as well as in the backcross males with the same sex chromosome combination, shows that this is not a reciprocal effect: the *subobscura* X in the balanced or mainly *subobscura* autosomal background does not form *esc*. The existence of a few exceptions in this type of F_1 male, with a lower degree of penetrance, is indicative of a threshold in the expressivity of the character. We do not have any data on the formation of the *esc* phenotype when a *subobscura* X interacts with a *madeirensis* Y and autosomal background. It should be mentioned that this kind of cross is extremely difficult to perform, since *subobscura* females hardly produce any female progeny when crossed to *madeirensis* males. It is even harder to obtain such a female that is viable and fertile so that it can produce progeny.

In the most productive cross (*madeirensis* females to *subobscura* males, F_1 females to *subobscura* males), only a small proportion (15 %) of the B_1 males carrying a *madeirensis* X display *esc*. We have searched for the possible influence of the autosomes (all except the dot) in the formation of *esc*, using three electrophoretic markers (Idh, Me, and Pept-3) and seven visible mutant markers. The results are shown in Table 1. Here we report only the detailed analysis of the Pept-3, a marker located on the E chromosome, which shows a significant influence.

	esc	normal
E mad/sub	34	101
E sub/sub	17	135

χ^2=9.57 for 1 df; significant P<0.01

From Table 1 it is clear that chromosomes J and O do not exert any influence. The situation is less clear for the U chromosome. While the marker net indicates no involvement of this chromosome, this marker is located near the distal end, and we do not have any information regarding the rest of the chromosome. Chromosome E, exemplified by three markers, does strongly influence the appearance of the *esc* phenotype: individuals heterozygous for the E chromosome (mad/sub), display *esc* more often than homozygous (sub/sub). Thus the *esc* seems to be expressed predominantly in males when a *madeirensis* X coexists with a heterozygous E chromosome.

Why are the backcross males found at a frequency of 15 % rather than 25 % as expected for two independently segregating factors (chromosomes A and E)? Is it possible that we have failed to identify a third independently segregating locus? Can the character be produced by several other chromosomal regions with small (not 'all or none') effect? We do not have any definite answer to any of these possibilities.

Extra sex combs and sterility/inviability

All the backcross males with extra sex combs had *madeirensis* X chromosome; these males also carried *subobscura* Y chromosome (females F_1 were crossed to *subobscura* males). We have shown that the incompatibility between sex chromosomes always results in the sterility of their carrier and also that backcross males with a *madeirensis* X on the hybrid background are less viable than those with *subobscura* X (KHADEM and KRIMBAS, in press). Therefore, all the backcross males from this given cross with extra sex combs are sterile and less viable than the normal males (without extra sex combs).

Abnormal head shape

Female and male hybrids display an abnormal head shape that varies in both degree and frequency among crosses. This phenotype could be described as a protuberance of the frontal part of the head and a bulging of the eyes.

All F_1 flies from *madeirensis* females crossed to *subobscura* males display the abnormal head. F_1 females are more severely affected than F_1 males, but the severity varies among crosses and also among repetitions of the same cross. In the reciprocal cross (*subobscura* females to *madeirensis* males), the F_1 females all show the abnormality but only some of the males display the character, varying from 10 % to 50 % in the different repetitions of the same cross.

Table 1. Results of the chi-square tests for independent segregation of the electrophoretic and visible markers with the segregation of the extra sex comb phenotype in backcross males. Two experiments with ma int are not consolidated (no homogeneous results) and are indicated separately. N is the total number of individuals examined.

ns indicates non-significant departure, two asterisks, a significant departure at 1 %

Marker	N	χ^2 (1 df)	Significance
chromosome U			
net	381	0.62	ns
chromosome E			
Pept-3	287	9.57	**
pp-pl	299	7.32	**
chromosome J			
Idh	183	2.21	ns
ma, int 1	533	0.26	ns
ma, int 2	713	3.08	ns
chromosome 0			
Me	214	0.31	ns
ch, cu	103	1.89	ns

2075 backcross males were classified according to the origin of their X chromosome, using antennae colour and its segregation from abnormal head shape in the usual cross, i.e., F_1 females (from the female *madeirensis* by male *subobscura*) crossed by male *subobscura*. The results are the following:

	abnormal face	normal face
sub X chrom.	225	1526
mad X chrom.	146	178

χ^2 = 192.76 for 1 df, P<0.001

This result indicates that the *madeirensis* X considerably increases the probability of formation of an abnormal head shape, which is found in nearly 50 % of the males carrying it. On the other hand, males with a *subobscura* X display this abnormality in nearly 12.5 % of the cases.

The segregation of the X chromosome from the head abnormality was also studied in the B_1 females. These females are produced by the same cross as the one reported above for their male broth-

ers. To analyse the females we have used two chromosomal markers, one is inversion *16BCD* located at the distal end of the *madeirensis* X, and the other is inversion *mad 1* at the proximal end. The results are shown below.

Inversion *16BCD*	abnormal face	normal face
sub/sub X chrom.	7	10
sub/mad X chrom.	8	1

$\chi^2 = 6.24$ for 1 df, $P<0.02$

Inversion *Mad 1*	abnormal face	normal face
sub/sub X chrom.	11	11
sub/mad X chrom.	4	0

$\chi^2 = 4.80$ for 1 df, $P<0.05$

These results indicate that both X markers do not segregate independently from the character. Nearly all the females heterozygous for inversions *Mad 1* and *16BCD* (mad/sub) have the head abnormality, whereas only half of them are abnormal when homozygous for *subobscura* X (sub/sub).

The influence of the autosomes (except dot) on this character is illustrated in Table 2. These data are based on the segregation of several genetic markers and the shape of the head in the backcross progeny. It is evident (Table 2) that visible markers located in chromosome E do not segregate independently of the abnormal head. J and O only in the case of the females progeny, and U both in females and males do segregate independently of the abnormal head shape. It is important to note that visible mutations can reduce severely viability of the hybrids. Their effect on the viability also depends on the genetic constitution of the individuals. It seems likely that this is happening, since there is no homogeneity between male and female segregations (viability and interactions differ according to sex). Thus a significant departure from a non-random segregation of the visible markers from the abnormal head shape should be taken with some caution.

Electrophoretic markers on the other hand do not seem to be subjected to such a complication. The simultaneous segregation of the three electrophoretic markers (in chromosomes E, J, and O), from the head abnormality is reported in Table 2. Pept-3 (E) and Me (O) indicate that chromosomes

Table 2. Chi-square test for independent segregation of the electrophoretic and visible markers from the abnormal head shape in females and males. N is the total sample size

ns indicates non-significant departure, two asterisks, a departure at 1 %, and three a departure at 0.1 %

Markers	N	χ^2	df	Significance
1. chromosome U				
net ♀♀	543	0.65	1	ns
♂♂	381	0.32	1	ns
2. chromosome E				
pp, pl ♀♀	755	33.87	3	***
♂♂	299	15.42	3	**
3. chromosome J				
ma int ♀♀	606	2.43	3	ns
♂♂	426	18.00	3	**
4. chromosome O				
ch cu ♀♀	106	2.32	3	ns
♂♂	67	12.11	3	**
Pept-3 (E) ♂♂	561	21.51	1	***
Idh (J) ♂♂	561	3.58	1	ns
Me (O) ♂♂	561	15.34	1	**
Interactions				
Pept-3–Idh	561	0.00	1	ns
Pept-3–Me	561	0.98	1	ns
Idh–Me	561	0.69	1	ns

E and O play an important role in determining this abnormality. The detailed data for each of the three electrophoretic markers are as follows.

		abnormal face	normal
Chrom. E (Pept-3)	mad/sub	91	209
	sub/sub	37	224
Chrom. J (Idh)	mad/sub	72	215
	sub/sub	56	218
Chrom. O (Me)	mad/sub	85	200
	sub/sub	43	233

There is no significant interaction between the three markers (Table 2). These results coincide, except the J chromosome, with those of the visible markers (we interpret the significant effect of the J chromosome, observed only in males, using visible markers, is due to lower viability of males carrying these mutations). Therefore, the abnormal head shape appears to be a product of an interaction between the A, E, and O chromosomes. The simplest explanation is as follows: all females with heterozygous X chromosome (sub/mad) show the abnormal face, the homozygous females (sub/sub) display the character in about 50 % of individuals

depending on the factors in the E and O autosomes being heterozygous (sub/mad). Males with the X of *madeirensis* show the abnormality in half of the cases according to the genotype of their two autosomes E and O. Males with the X of *subobscura* show the character only if both their autosomes E and O are heterozygous, and even then only half of the males display it.

One experiment gave a discordant result. Exceptionally, the F_1 males from *D. madeirensis* females crossed to *D. subobscura* males had a normal head shape but white antennae (instead of having abnormal head shape and white antennae). Their sisters, however, had the abnormal head. These females crossed to *subobscura* males produced backcross males, some of which had the abnormal head. The backcross males were analyzed for the three electrophoretic markers, and independent segregation was observed for all the markers:

(Pept-3 χ^2=2.28 1df N=287; Idh χ^2=0.55 1df N=183; and Me χ^2=2.38 1 df N-214).

This perhaps points to the possible existence of a type of *madeirensis* X more compatible with a normal head shape in a heterozygous background. If this assumption is correct, it can be concluded that the X chromosome plays a decisive role: that is to say, there is an interaction between the X chromosome on one hand and the E and/or O chromosome on the other hand. The lack of electrophoretic markers diagnostic of the two species and located in the X chromosome has not permitted us to test this hypothesis.

Abnormal head shape and sterility/inviability

All the backcross males with abnormal head shape carrying *madeirensis* X are sterile (X/Y incompatibility). The possibility remains for the abnormal males with *subobscura* X to be fertile. To test this possibility we put 128 abnormal backcross males individually together with two virgin females of *D. subobscura* and kept them for two weeks. In none of the cases was progeny produced. Thus abnormal males were considered to be sterile, judged by their inability to produce offspring. Flies with severe abnormality of head have a low viability and they usually die few days after eclosion, many more also die as pupae. In one experiment, out of 955 males that died in the pupal stage 891 had abnormal head shape (93 %). The same experiment resulted in 2075 adult males, of which 371 had head abnormality (18 %). These data are indicative of the lower viability of the backcross hybrid males with abnormal head shape.

Extra sex combs and head abnormality

These two characters are positively correlated. Abnormal head shape accompanies extra sex comb phenomena from 34 % to 86 % of the times, depending on the stock used or the repetitions of the same cross. The genetic analysis of these two characters also showed resemblance (i.e., involvement of the X and E chromosomes in both cases).

Discussion

Two abnormal phenotypes have been analysed in backcross hybrids between *D. madeirensis* and *D. subobscura*: (1) extra sex comb, which is shown to be a product of an interaction between the X and E chromosomes; and (2) abnormal head shape, which is a product of an interaction between the X, E, and O chromosomes. In both cases, the X chromosome has a preponderant effect in the formation of abnormal phenotypes. The X chromosome also plays a major role in fertility and viability of the hybrids. Apart from the X chromosome other autosomal factors have an influence in hybrid abnormalities in addition to sterility and viability. For sterility and viability, the influence of autosomes seems to be additive and not all or none type like the X chromosome (KHADEM and KRIMBAS, in press).

It is important to note that the validity of these results may only hold under the given conditions of the experiment (i.e., type of crosses performed). The two abnormal characters appear in the progeny of all different types of crosses between the two species of *D. madeirensis* and *D. subobscura*. The expression and percentages differ from one type of cross to another. The genetic mechanisms underlying these abnormalities have not yet been determined in all crosses. Most of the results reported in the present paper are from *D. madeirensis* females crossed to *D. subobscura* males and the F_1 progeny crossed to *D. subobscura* males. *Subobscura* has been used as the male parents for the following reasons: (1) the mutant markers only exist in this species; (2) performing the reciprocal cross, i.e., *subobscura* females by *madeirensis* males is an extremely difficult task, mating does take place but most of the progeny dies at a very early stage of development. The surviving progeny consists mainly of sterile males and only a very few females

(10 females–175 males). Moreover, most of these females have a very low viability and die a few days after their eclosion (only one out of the 10 F_1 females lived long enough to produce progeny). It is hard to determine if most of the females are also sterile or not, since they do not live long enough to develop mature eggs (their ovaries remain underdeveloped at the time of death).

In spite of these difficulties, the hybrids between the two species of *D. madeirensis* and *D. subobscura* offer very good opportunities to study the genetic mechanism(s) underlying developmental incompatibility between the two species. Morphological characters have been studied in some other pairs of species. Most of these studies indicate the polygenic nature of the morphological differences (TEMPLETON 1977; LAND 1981; COYNE 1983). However, other results show that some interspecific differences are caused by a small number of genes (BRYANT and CARSON 1979; KRIMBAS and LOUKAS 1984).

In the present study we have used many markers scattered in different regions of different chromosomes (except U). We cannot rule out the possibility of an influence exerted by the U chromosome. In the present study, the X chromosome influence is much more pronounced than that of the autosomes. This is not the case in genetic analyses of morphological differences between the species (see CHARLESWORTH et al. 1987). In this respect, however, the two abnormalities we describe should not be taken as neutral morphological characters. They affect fitness, being correlated with sterility and lower viability, as it was mentioned earlier. It is not clear whether the developmental abnormalities are the cause of fitness deterioration or their correlation to fitness is due to the fact that in both cases the X chromosome plays a major role.

Most of the morphological characters studied, except the testis size, show a quantitative difference in the two parental species. Some quantitative morphological characters were also studied in the hybrids between *D. madeirensis* and *D. subobscura* (KRIMBAS and LOUKAS 1984): the characters were the number of teeth in male claspers and teeth in the sex combs. The numbers of genes involved in these cases were estimated as one or two in both cases but no chromosome or chromosomal regions were identified.

In the present paper and those of WEISBROT (1963) and ORR (1990) the characters studied are characteristic of the hybrids. The abnormality in tergite development in hybrids between *D. pseudoobscura* and *D. persimilis* is caused by many factors distributed on all of the chromosomes (WEISBROT 1963). Considering that these two species are morphologically indistinguishable it was concluded that the differences between species may be extensive even when the phenotypes are very similar. ORR (1990) has identified maternal effect and loss of microchromosome as the cause of some abnormalities in hybrids between *D. virilis* females and *D. lummei* males.

Our results indicate that the genetic bases of the two abnormal characters (extra sex combs and abnormal head shape) resemble each other as well as do the genetic bases of the sterility and inviability of the hybrids between *D. madeirensis* and *D. subobscura*.

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