Colour polymorphism in *Drosophila mediopunctata*: genetic (chromosomal) analysis and nonrandom association with chromosome inversions

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The presence of three dark spots on the abdomen is typical of the *tripunctata* group of *Drosophila*, which is the second largest Neotropical group, with 56 species. In some species, such as *D. mediopunctata*, the colour pattern varies considerably: ranging from flies showing no spots up to flies with three dark spots. In this paper, we present a genetic (chromosomal) analysis of this character showing that this colour polymorphism is genetically determined mainly by the second chromosome. Since this chromosome is the most polymorphic for inversions in this species, we also examined the influence of the inversions on this character. We used strains in which different second chromosomes were placed

on the same genetic background and the offspring between them. We found a nonrandom association between the number of spots and the inversions *PA0* and *PC0*. Thus, our results are consistent with the idea that the factors or genes determining a conspicuous polymorphism are likely to be associated, forming a supergene, and this association would be most efficiently accomplished through a chromosome inversion. Moreover, this is the first time that an association between a conspicuous morphological polymorphism and chromosome inversions has been described.

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Introduction

The adaptive nature of chromosome inversions in *Drosophila* has been accepted since Dobzhansky's classic work (Wright and Dobzhansky, 1946), which first established that different chromosomal arrangements were under natural selection.

As no recombinants are produced between genes within different inversions – crossingover is suppressed within the inverted region in inversion heterozygotes – Dobzhansky (1970) believed that inversions could be considered supergenes, which accumulated coadapted alleles. Ford (1964) thought that including coadapted alleles in a chromosome inversion was the most effective way to ensure that they would segregate as a block, to produce only high fitness phenotypic variants. Ford also proposed that polymorphic adaptations occurred mainly through a genetic architecture in which genes are tightly linked so that they act as a single unit. Inversion polymorphisms in *Drosophila* have been associated with several fitness-related characters (Rodríguez *et al*, 1999), behaviour (Dahlgaard *et al*, 2001) and morphological traits (Izquierdo *et al*, 1991; Bitner-Mathé *et al*, 1995; Bertrán *et al*, 1998). Recently there has been a resurgence of interest in the significance of chromosomal inversions in evolution

and speciation. Noor *et al* (2001) studied the genetic basis of hybrid sterility between *Drosophila pseudoobscura* and *D. persimilis*. They concluded that inversions could create linkage groups that cause sterility between hybridising taxa which would favour reproductive isolation. It has also been proposed that the reduction in recombination caused by chromosome inversions could act synergistically with isolation genes and increase the plausibility of certain models of speciation (Rieseberg, 2001).

There are presently about 2000 known species of *Drosophila* (Powell, 1997). Yet, only a few cases of colour polymorphism have been examined in this genus. One of the best studied cases is *D. polymorpha*. da Cunha (1949) identified one locus and two alleles (with no dominance) that determined the abdomen colour pattern polymorphism in this species. Heed and Blake (1963) later found another allele, and Martinez and Cordeiro (1970) discovered that modifiers segregated independently of the major locus. In *D. kikkawai*, abdominal colour polymorphism is determined by one locus and two alleles (Gibert *et al*, 1999), whereas in *D. lebanonensis*, the colour of the thorax is controlled by one locus (two alleles) and modifiers (Pipkin, 1962).

The *tripunctata* group is the second largest Neotropical group of *Drosophila* in number of species, with 56 described species (Vilela, 1992). It is particularly abundant in forested areas of southern Brazil during the winter (Saavedra *et al*, 1995). The group name *tripunctata* is derived from the presence of three dark spots on the last tergites of the abdomen. However, not all species show this trait. In some species, there is only a dark band

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on the last tergite instead of three spots, while in others there is intraspecific variation (Frota-Pessoa, 1954). This is the case in *D. mediopunctata*, a species distributed from southern South America to Central America. The abdominal colour pattern in *D. mediopunctata* varies considerably, from no spots to three dark spots, on the fourth, fifth and sixth abdominal tergites. In addition to number, these spots also vary in size. Males tend to have more and bigger spots than females (Frota-Pessoa, 1954).

D. mediopunctata has six pairs of chromosomes: five acrocentrics and a dot that does not undergo polytenisation. The X, second and fourth chromosomes are polymorphic for inversions. The second chromosome is the most polymorphic and its inversions can be divided into two groups according to the region as follows: there are eight inversions in the distal region (*DA*, *DP*, *DS*, *DV*, etc) and nine in the proximal region (*PA0*, *PB0*, *PC0*, *PC1*, etc) (Ananina et al, 2002). There is intense linkage disequilibrium between distal and proximal inversions, for example, *DA* is associated with *PA0*, with a D' value of 0.98; *DP* is associated with *PC0* ($D' = 0.97$); and *DS* with *PC0* ($D' = 0.95$) (Peixoto and Klaczko, 1991).

In this paper, we present a genetic (chromosomal) analysis of the variation of the number of abdominal spots in *D. mediopunctata*. We show that this colour polymorphism is genetically determined mainly by one chromosome (the second chromosome). Examination of the influence of inversions of this chromosome on the character reveals a nonrandom association between inversions *PA0* and *PC0* and the number of spots. To our knowledge, this is the first time a conspicuous morphological polymorphism has been found to be associated with chromosome inversions.

Materials and methods

The colour polymorphism

The number of spots was counted on flies that were at least 4 days old, by which time the colour intensity had stabilised. The number of spots varied, with flies possessing no spots, one (on the sixth tergite), two spots (on the fifth and sixth tergites) and three spots (on the fourth, fifth and sixth tergites) (Frota-Pessoa, 1954). In flies with three spots, the spot on the fourth tergite sometimes merges with the posterior pigmented band. In these cases, the phenotype was scored as '3D', but the entire analysis was carried out by considering this pattern as having three spots.

Strains used in the genetic analysis

The following strains were used:

- *ITC-29I*: A strain selected for zero spots and brother-sister crossed for over 20 generations.
- *NA*: A marker strain (Carvalho and Klaczko, 1993) carrying the visible mutations Δ (*Delta*), *Im* (*Impar*), *cr* (*coral*) and *al* (*alfinete*) on the second, third, fourth and fifth chromosomes, respectively, and sharing its X and Y chromosomes with strain CR27A.
- *CR27A*: A marker strain carrying the dominant visible mutations Δ -5 (*Delta*-5) on the second chromosome, and the recessive mutations *cb* (*cabernet*), *cr* (*coral*) and *al* (*alfinete*) on the third, fourth and fifth chromosomes, respectively. This strain shares its X and Y chromosomes with strain CR27B.

- *CR27B*: A marker strain carrying the visible mutations *cb* (*cabernet*), *cr* (*coral*) and *al* (*alfinete*) on the third, fourth and fifth chromosomes, respectively, and sharing its X and Y chromosomes with strain CR27A.

In strain ITC-29I, the zero spot phenotype was fixed whereas in the remaining strains the three spots phenotype was fixed.

Marker strains used to produce strains with the same genetic background

The following strains were used:

- *CR26A*: A marker strain carrying the recessive mutations *mt* (*merlot*), *cr* (*coral*) and *al* (*alfinete*) on the second, fourth and fifth chromosomes, respectively. This strain shares its X and Y chromosomes with strains CR26B, CR26C and CR26J. The second chromosome karyotype is *DV-PC0/DV-PC0*.
- *CR26B*: A marker strain carrying the dominant visible mutation Δ (*Delta*) on the second chromosome, and the recessive mutations *mt* (*merlot*), *cr* (*coral*) and *al* (*alfinete*) on the second, fourth and fifth chromosomes. The second chromosome karyotype is *DV-PC0/DV-PC0*.
- *CR26C*: A marker strain carrying the dominant visible mutation *Im* (*Impar*) on the third chromosome, and the recessive mutations *mt* (*merlot*), *cr* (*coral*) and *al* (*alfinete*) on the second, fourth and fifth chromosomes, respectively. The second chromosome karyotype is *DV-PC0/DV-PC0*.
- *CR26J*: A marker strain carrying the dominant visible mutation Δ -5 (*Delta*-5) on the second chromosome, and the recessive mutations *mt* (*merlot*), *cr* (*coral*) and *al* (*alfinete*) on the second, fourth and fifth chromosomes, respectively. The second chromosome karyotype is *DA-PA0/DV-PC0*.

Strains CR26A, CR26B, CR26C and CR26J share their sex chromosomes.

Strains with different second chromosomes on the same genetic background

To test whether there was a nonrandom association between second chromosome inversions and the phenotypes of dark abdominal spots, we produced strains that differed from each other on the second chromosome but shared the same genetic background. We used isofemale lines obtained from two collections made in Serra do Japi, SP, Brazil (23°11'S, 46°40'W) in July 1994 and May 1995. The females were crossed with marker strains (CR26A, CR26B, CR26C and CR26J) and the second chromosome was then made autozygous by inbreeding (Figure 1).

Females from the isofemale line were initially crossed with males from strain CR26C (Figure 1). From the offspring, we selected males with phenotype *Impar*, which were heterozygous for the recessive markers *mt*, *cr* and *al*, and for the dominant marker *Im*. These males were individually crossed with females from strain CR26A and males with the phenotypes *Impar*, *coral* and *alfinete* were selected from the offspring. Thus, the fourth and fifth chromosomes from the isofemale line were replaced by the corresponding marked chromosomes. The selected males were crossed with females

Figure 1 Crosses made to obtain strains with different second chromosomes on the same genetic background. All original chromosomes from the isofemale line were replaced, except for the second chromosome, which was made autozygous. The chromosomes from the isofemale line are in bold.

CR26A and the larvae from these crosses were karyotyped. If the karyotype was *DA-PA0*, males with the phenotypes *Impar*, coral and alfinete were crossed with females from strain CR26B. This strain carries mutations Δ and *mt* in a chromosome with a haplotype *DV-PC0*. Thus, in the progeny we are able to select double heterokaryotypes (*DA-PA0/DV-PC0*) to avoid recombination. If the karyotype was *DP-PC0*, *DS-PC0* or *DV-PC0*, males with the same phenotype were crossed with females from strain CR26J. This strain carries mutations Δ -5 and *mt* marking the haplotype *DA-PA0*, and as, in the other cross, one may select double heterokaryotypes (eg *DP-PC0/DA-PA0*) avoiding recombination. In both cases, delta, alfinete and coral males and females of the offspring would form the next cross. The flies produced in this cross that were only coral and alfinete were used to start the new strain. If the second chromosome from the isofemale line was lethal, males and females delta were crossed and the strain was kept balanced against the *Delta* or *Delta*-5 mutation, which is also lethal.

Using this approach, we obtained strains that were differentiated in the second chromosome, but not in the other chromosomes, that is, they shared the same genetic background. Overall, 25 strains (14 *PA0* and 13 *PC0*) were produced.

Genetic analysis

To carry out the genetic analysis of the colour polymorphism in *D. mediopunctata*, we used the same experimental strategy as Spicer (1991) when he analysed the differences in pigmentation between *D. virilis* and *D. novamexicana*. We first examined the effect of the sex chromosomes by reciprocal crosses, and then analysed the effects of the autosomes using visible mutants marking each autosome.

Detection of the effect of the sex chromosomes: Reciprocal crosses were carried out between strains ITC-29I and NA to detect the presence of genetic factors that could determine the colour polymorphism on the sex chromosomes. If the X or Y chromosome affected the character, then male offspring of one cross should be different from the males of the other. The female offspring served as a control and were expected to have the same distribution of phenotypes since they had the same autosomes and sex chromosomes in both reciprocal crosses. This would not happen if there was a maternal effect or a maternal inheritance.

Genetic (chromosomal) analysis: Females from strain ITC-29I (phenotype 0, for dark spots on the abdomen) were crossed with males from strain CR27A. This strain

is fixed to phenotype 3 and has a recessive lethal mutation with a dominant visible effect (*Delta-5*, $\Delta-5$), heterozygous on the second chromosome. The strain is homozygous for recessive mutations on the third, fourth and fifth chromosomes. Male offspring with phenotype Δ were backcrossed with strain CR27B. This strain carries the same mutations on the third, fourth and fifth chromosomes as CR27A, but is homozygous for the wild allele of $\Delta-5$. The offspring were raised under controlled conditions of temperature (16.5°C) and density (20 larvae/vial). Since there is no crossingover in males of *D. mediopunctata*, it is possible to assess the influence of each of the marked chromosomes. If the distribution of phenotypes is independent of the genotype of the markers, then there is no influence of the chromosome; otherwise, the chromosome carries at least one factor that determines the character.

Strains carrying different inversions on the same background

First instar larvae were collected from each strain and 15 larvae per vial were kept at 20°C with culture medium (16% yeast, 2% sugar and 2.5% agar) until all adults had emerged.

The patterns of abdominal spots were analysed in only 10 strains for each karyotype, since the second chromosome of the remaining strains carried at least one lethal gene, which made it impossible to examine individuals autozygous for this chromosome. All strains carrying *PA0* in the proximal region were *DA* in the distal region of the chromosome, whereas strains carrying *PC0* carried *DV*, *DS* or *DP* in the distal region.

The statistical analysis was carried out using an ANOVA of the means of the number of spots per individual for each sex of each strain, to allow assessment of the effects of the second chromosome karyotype, sex and the interaction between these two factors. The advantage of using the means of each strain instead of the number of spots for each individual is that, as sample size increases, the means approach the normal distribution (Sokal and Rohlf, 1995).

Crosses between strains with the same genetic background

Since autozygosis for an entire chromosome is quite unlikely in nature, we planned crosses between strains with the same karyotype, so that the resulting offspring would simultaneously be homokaryotypic and allozygous (two alleles of independent origin) for the proximal inversions of the second chromosome. The crosses were chosen randomly among all possibilities, with the condition that each strain would participate in only two crosses. In this way, the number of crosses was equal to the number of strains available and we did not inflate the sample size.

Since it was not possible to make all of the crosses at once, they were made in sets of five. One of these crosses was repeated each time a new set of crosses was made, in order to confirm that there were no changes in the environmental conditions during the experiment.

Crosses were made with males from one strain and females from another strain. Reciprocal crosses were not carried out. Again, first instar larvae were collected from the offspring of each cross and placed in vials containing

10 ml of culture medium. These larvae were kept under four conditions: 12 or 96 larvae per vial, at 20°C, and 12 or 96 larvae per vial, at 16.5°C.

The effect of each of these variables (karyotype, temperature, density and sex) on the colour polymorphism was tested by ANOVA, in a manner analogous to the analysis of the strains.

Results

Genetic analysis

Effect of the sex chromosomes: There were no significant differences between the results of the reciprocal crosses between strains NA (pure 3) and ITC-29I (pure 0). Therefore, sex chromosomes had no significant effect (Table 1).

Autosomes: Genetic analysis revealed that the second chromosome was the most important chromosome in determining the colour polymorphism. Figure 2 shows that the phenotypes changed considerably, depending on the origin of the second chromosome (compare the lower and upper halves of each graph in the figure). In addition, ANOVA (Table 2) revealed significant effects of sex and the fifth chromosome; the third chromosome exerted an effect that bordered on significance ($P=0.088$). ANOVA detected no significant effects for any of the interactions (not shown).

Nonrandom association between karyotype and colour pattern

Strains with different second chromosomes on the same genetic background: Figure 3 shows that strains in which the karyotype was *PA0* usually had fewer spots than those in which the karyotype was *PC0*. The difference between the two karyotypes was significant, as was the difference between sexes. There was no significant interaction between these two factors (Table 3).

Crosses: The karyotype of the second chromosome had a significant effect on the phenotype of the colour polymorphism among the offspring of crosses between strains with the same genetic background for this chromosome (Table 4). Figure 4 shows that crosses between strains carrying *PA0* tended to produce offspring with fewer abdominal spots than crosses between strains carrying *PC0*.

Table 1 Numbers of flies from each phenotype in reciprocal crosses

Cross	Sex	Number of spots				Total
		0	1	2	3	
1	Males	0	60	36	8	104
	Females	0	127	1	0	128
2	Males	0	42	31	3	76
	Females	0	83	2	2	87

Reciprocal crosses: 1 – females NA and males ITC-29I; 2 – females ITC29-I and males NA (Fisher's exact test, $P=0.15$ and 0.49 for females and males, respectively).

Figure 2 Percentages of the patterns of colour polymorphism based on genetic analysis showing the effects of each chromosome on males and females separately. In each chart, the effect of the second chromosome becomes evident when the upper half (where the second chromosome is a heterozygote from a pure 0 strain and a pure 3) is compared with the lower half (where the second chromosome is a homozygote from a pure 3 strain).

Table 2 ANOVA for the data used to assess the effects of each autosome and sex on the number of abdominal spots

Source	Sum of squares	df	Mean square	F-ratio	P-value
Chromosome II	64.440	1	64.440	179.932	<0.001
Chromosome III	0.514	1	0.514	1.435	0.231
Chromosome IV	1.045	1	1.045	2.918	0.088
Chromosome V	2.180	1	2.180	6.087	0.014
Sex	2.184	1	2.184	6.099	0.014
Error	367.087	1025	0.358		

Other effects: Temperature had a significant effect and was the source of most of the variation (Table 4), leading to flies with more spots at lower temperatures. The effect of sex was also significant so that males tended to have more spots than females (Tables 5 and 6). In addition,

Figure 3 Percentages of the patterns of colour polymorphism in strains with different second chromosomes on the same genetic background. In each pair of bars, the upper bar indicates the proportions of the phenotypes among the females and the lower bar refers to males. Each strain is identified by a letter on the left side of the corresponding bars.

Table 3 Results of the ANOVA applied to the average number of spots per individual of each strain to assess the effects of karyotype, sex, strain (nested by karyotype) and interaction between genotype and sex

Source	Sum of squares	df	Mean square	F-ratio	P-value
Karyotype (K)	6.167	1	6.167	68.743	<0.001
Sex (S)	0.938	1	0.938	10.458	0.005
Strain (karyotype)	14.546	16	0.909	10.135	<0.001
K × S	0.064	1	0.064	0.710	0.412
Error	1.435	16	0.090

Figure 4 Patterns of colour polymorphism for crosses with different second chromosomes on the same genetic background. The data are for flies raised in 20°C at density of 12 larvae per vial. In each pair of bars, the upper bar indicates the proportions of the phenotypes among the females and the lower bar refers to males. The letters on the left of the bars identify each cross, corresponding to the strains in Figure 3 (for example, N × E is the cross between strains N and E from Figure 3).

there were significant interactions between karyotype and temperature, and between sex and temperature, but we did not detect any significant effect of density (Table 4).

Control cross: The phenotype of the cross used as the control was particularly favourable because it was

Table 4 Results of the ANOVA applied to the average number of spots in each cross, to assess the effects of karyotype, density, temperature, sex, cross (nested by karyotype) and all possible interactions

Source	Sum of squares	df	Mean square	F-ratio	P-value
Karyotype (K)	6.388	1	6.388	53.725	<0.001
Density (D)	0.026	1	0.026	0.215	0.644
Temperature (T)	34.804	1	34.804	292.709	<0.001
Sex (S)	2.587	1	2.587	21.753	<0.001
Cross (karyotype)	21.609	23	0.940	7.902	<0.001
K × D	0.067	1	0.067	0.563	0.454
K × T	2.856	1	2.856	24.017	<0.001
K × S	0.031	1	0.031	0.257	0.613
D × T	0.018	1	0.018	0.148	0.701
D × S	0.001	1	0.001	0.008	0.931
T × S	0.968	1	0.968	8.142	0.005
K × D × T	0.010	1	0.010	0.085	0.770
K × D × S	0.003	1	0.003	0.024	0.876
K × T × S	0.049	1	0.049	0.412	0.522
D × T × S	0.002	1	0.002	0.017	0.897
K × D × T × S	0.007	1	0.007	0.060	0.808
Error	18.906	159	0.119		

intermediate at 20°C. This cross produced offspring in which neither of the extreme phenotypes (0 and 3) was the most common, thus allowing us to detect any displacement of the phenotype caused by environmental changes.

The use of a control cross ensured that we would detect any change in the environmental variables during the course of the experiment. This was indeed seen: significant differences between the offspring of control crosses conducted on different dates were observed (ANOVA, not shown). Thus, the crosses were reanalysed, using the logarithm of the ratios between the mean of each cross and the mean of the corresponding control cross, considering the date, sex, density and temperature. In this way, the means of the crosses were standardised to the control, in order to eliminate all environmental effects, including the influence of the factors mentioned above. This second analysis (not shown) reduced the effects of temperature and density, which became nonsignificant whereas the effect of sex remained. The interactions between karyotype and temperature, and sex and temperature also persisted and a new, marginally significant ($P=0.049$) interaction (between density and sex) appeared.

The effect of karyotype remained highly significant and became the factor with the highest influence on the character ($F=48.52$; $P=0.001$). Thus, the mathematical correction diminished the environmental effects and emphasised the difference between the two karyotypes.

Table 5 Average number of spots per offspring in crosses between strains PA0, in relation to temperature and larval density

Cross	Sex	16.5°C				20°C			
		12 larvae		96 larvae		12 larvae		96 larvae	
		n	Average	n	Average	n	Average	n	Average
I × L	M	22	3	11	3	48	1.23	42	1.17
I × L	F	16	3	9	3	38	1.03	29	1.14
H × N	M	24	3	29	3	49	2.08	30	2.00
H × N	F	28	3	14	2.86	51	1.18	24	1.25
F × B	M	10	3	13	2.92	30	1.27	5	1.20
F × B	F	6	2.67	14	2.71	44	1.02	6	0.83
C × I	M	27	3	9	3	49	2.06	29	1.93
C × I	F	35	3	15	3	43	1.51	40	1.38
J × G	M	26	3	11	3	41	2.90	21	3.00
J × G	F	16	3	17	3	63	2.56	34	2.59
A × C	M	23	3	9	3	44	2.27	18	2.50
A × C	F	33	3	22	3	39	1.85	20	1.95
K × H	M	12	3	16	3	45	1.38	31	1.81
K × H	F	19	2.68	32	2.63	39	1.05	42	1.00
M × E	M	25	2.64	25	2.80	25	1.20	34	1.24
M × E	F	19	2.68	21	2.57	33	1.03	20	1.00
N × E	M	30	3	4	2.75	22	2.77	10	2.50
N × E	F	26	2.92	17	3	17	1.65	13	2.00
F × J	M	28	2.93	134	2.76	88	2.42	26	1.69
F × J	F	35	2.69	66	2.23	92	2.03	30	1.20
G × L	M	30	3	90	3	50	2.82	35	2.91
G × L	F	46	2.96	60	3	75	2.75	42	2.88
B × M	M	20	2.30	62	2.34	56	2.38	18	2.06
B × M	F	31	2.58	70	2.34	33	1.18	38	1.74
A × K	M	25	2.72	40	2.68	31	2.26	41	1.88
A × K	F	26	2.73	62	2.52	86	2.08	46	1.07
Males Total		302	2.89 ± 0.06	453	2.87 ± 0.06	578	2.08 ± 0.17	340	1.99 ± 0.17
Females Total		336	2.84 ± 0.05	419	2.76 ± 0.08	653	1.61 ± 0.17	384	1.54 ± 0.18

Table 6 Average number of spots offspring for crosses between strains PC0, in relation to temperature and larval density

Cross	Sex	16.5°C				20°C			
		12 larvae		96 larvae		12 larvae		96 larvae	
		n	Average	n	Average	n	Average	n	Average
W × Y	M	36	3	45	3	53	3	47	3
W × Y	F	33	3	56	3	60	2.92	43	2.91
X × Y	M	16	3	—	—	18	3	21	2.86
X × Y	F	23	3	—	—	32	2.97	39	3
W × X	M	35	3	30	3	61	3	38	3
W × X	F	27	3	38	3	77	3	50	3
Q × T	M	28	2.93	32	2.90	47	1.38	35	1.46
Q × T	F	34	2.36	30	2.53	46	1	29	1.03
P × S	M	30	3	25	3	84	2.69	29	2.69
P × S	F	28	3	51	3	73	2.70	30	2.13
S × U	M	25	3	26	3	48	2.96	35	2.94
S × U	F	38	3	25	3	50	2.58	39	2.44
T × O	M	53	2.89	15	3	50	1.88	37	2.05
T × O	F	46	2.59	15	2.73	51	1.17	42	1.33
V × P	M	36	3	34	3	33	2.70	35	2.20
V × P	F	39	3	45	3	43	2.30	35	2.09
U × R	M	39	3	28	3	33	2.09	44	2.11
U × R	F	42	2.98	56	2.95	54	1.56	46	1.28
Z × R	M	59	3	28	3	56	2.16	32	2.19
Z × R	F	61	2.75	34	2.79	51	1.75	22	2
Q × O	M	50	3	26	2.92	58	2.45	26	2.54
Q × O	F	64	2.89	31	2.87	54	1.83	31	2.13
V × Z	M	39	3	28	3	62	2.94	43	3
V × Z	F	46	3	40	3	66	2.74	38	2.92
Males Total		446	2.98 ± 0.01	317	2.98 ± 0.01	603	2.52 ± 0.15	422	2.50 ± 0.14
Females Total		481	2.88 ± 0.06	421	2.90 ± 0.04	657	2.21 ± 0.21	444	2.19 ± 0.20

Discussion

Genetic analysis

Chromosomal analysis is useful for identifying the sources of genetic variation in a character among chromosomes. This classic approach has been used in the genetic analysis of hybrid sterility between *D. pseudoobscura* and *D. persimilis* by Dobzhansky (1936). He concluded that the inheritance of the character was polygenic, with at least one locus on each chromosome. An almost identical genetic analysis to the one used here revealed the existence of several autosomal suppressors of *sex ratio* in *D. mediopunctata*, with at least one locus in each major autosome (Carvalho and Klaczko, 1993).

Several studies have investigated the genetics of differences in abdominal pigmentation between closely related species. Spicer (1991) – applying the same experimental design used here – showed that four autosomes and the sex chromosomes had significant effects on the abdominal colour differences between *D. virilis* and *D. novamexicana*, showing that at least five loci are involved. Hollocher *et al* (2000) studied two species of the *D. dunni* subgroup and analysed three areas of the abdomen individually. They showed that the species differences were due to both X chromosomes and autosomes, as well as maternal and paternal effects, depending on the abdominal area. Another genetic analysis between two closely related species – *D. yakuba* and *D. santomea* – revealed that at least three genes (each located on a different chromosome) in females and five genes in males affected the pigmentation differences

between the two species (Llopart *et al*, 2002b). In addition, the X chromosome was responsible for nearly 90% of the pigmentation differences. The difference between *D. americana* and *D. novamexicana* is also determined by at least one locus in the X chromosome and a moderate number of genes located in the autosomes (Wittkopp *et al*, 2003).

Colour polymorphisms have also been analysed in other groups, such as the butterfly *Bicyclus anynana* that has been extensively studied (for a review see Brakefield, 2003). This butterfly presents seasonal variation with respect to wing colour pattern (Brakefield and Reitsma, 1991). A genetic analysis of this trait concluded that at least five genes are involved in its determination (Wijngaarden and Brakefield, 2000).

As shown here, the colour polymorphism in *D. mediopunctata* was determined by genes localised on the second and fifth chromosomes. The influence of the second chromosome was much higher than for any of the other chromosomes, suggesting the existence of a major locus or several minor effect loci on this chromosome.

Even though the X chromosome seems to play an important role in the determination of the abdominal pigmentation differences between the studied species of *Drosophila*, we did not detect its influence on the colour polymorphism in *D. mediopunctata*.

Caveat: It is important to bear in mind that the genetic analysis we carried out can only detect differences between the strains analysed. Thus, there is the possibility that loci (located in other chromosomes),

which contribute to the determination of this character, were not detected because the strains used in our experiments carried the same alleles (or alleles for equivalent effects). To minimise this limitation, we used strains selected for the maximum difference between them.

Temperature, sex and density

The effect of temperature was very intense since it increased the number of spots at the lower temperature and was responsible for most of the variation in the results of crosses between strains with the same background. In *D. melanogaster* and *D. simulans*, Gibert *et al* (1996) also found a strong temperature effect on abdominal pigmentation, similar to that which we observed in *D. mediopunctata*. In *D. melanogaster* and *D. simulans*, the influence of temperature was similar on the trident pigmentation on the thorax (Capy *et al*, 1988). In *D. simulans*, the trident is evident only in flies raised at low temperatures. Although there is little phenotypic plasticity for pigmentation in *D. kikkawai*, a more intense pigmentation is seen in flies raised at lower temperatures (Gibert *et al*, 1999). Increased pigmentation as a result of lower developmental temperatures is often interpreted as an adaptation for thermoregulation (Capy *et al*, 1988; Gibert *et al*, 1996, 1999): darker flies absorb more radiation and heat faster, which is advantageous in a cold environment. However, according to Stevenson (1985), any radiant heat that is absorbed by an insect as small as a *Drosophila* will quickly be lost by convection. Based on this, Crill *et al* (1996) suggested a number of alternative explanations, including nonadaptive linkage with other genes that are adaptive at low temperature, strengthening of melanised cuticles that would help compensate for larger body size, increased protection against UV radiation and concealment from visual predators when the insect tries to raise its temperature by sitting on a dark object. Therefore, the association between developmental temperature and pigmentation in *Drosophila* needs further investigation.

The effect of sex was always significant with males tending to have more spots than females. These results are in agreement with those of Frota-Pessoa (1954), who observed that *D. mediopunctata* males usually had more spots, which also tended to be darker than in females. A significant interaction between sex and temperature was observed, suggesting that males and females have different reaction norms. Kopp *et al* (2000) showed that differences in the regulation of the *bric-a-brac* (*bab*) gene are responsible for the sexual dimorphism in the abdominal pigmentation of *D. melanogaster*. They also found that males discriminate against mutant females with a male pigmentation pattern – darker abdomen – but females do not show any preference between normal or mutant (ie with a female abdominal pigmentation) males, which led the authors to suggest that the less pigmented abdomen in females may be maintained by sexual selection. However Llopart *et al* (2002a) argued that this mechanism is unlikely to work under natural conditions (see also Stern, 2000).

Density was not an important factor determining the colour polymorphism. Freire-Maia (1964) suggested that the frequencies of the abdominal colouration phenotypes in *D. kikkawai* depend upon the conditions of the culture

medium and larval density. This does not seem to be the case with *D. mediopunctata*.

Chromosome inversions

Since the second chromosome, which is the most polymorphic in *D. mediopunctata* (Peixoto and Klaczko, 1991; Ananina *et al*, 2002), showed greater influence on the character, we examined the correlations between inversions and the number of abdominal spots, that is, whether different second chromosome karyotypes had different effects on the colour polymorphism phenotype.

Dobzhansky (1970) believed that the suppression of recombination caused by chromosome inversions would be advantageous if the inverted region carried a supergene, such that inversions that kept coadapted gene complexes together would be favoured by natural selection. Evidence for polymorphism conditioned by supergenes has been found in land snails (Murray and Clarke, 1976; Jones *et al*, 1977), ladybird *Adalia bipunctata* (Marples *et al*, 1993) and butterflies (Mallet *et al*, 1990; Gordon and Smith, 1998).

Since chromosome inversions can behave exactly like supergenes, as predicted by Ford (1964), the existence of polymorphisms would be expected to be determined by different chromosome inversions. Several studies have demonstrated the influence of *Drosophila* chromosome inversions on morphological characters such as wing size and shape in *D. mediopunctata* (Bitner-Mathé *et al*, 1995), extra bristles in *D. melanogaster* (Izquierdo *et al*, 1991), size-related traits in *D. buzzati* (Bertrán *et al*, 1998) and more recently in traits showing clinal variation (Weeks *et al*, 2002; Calboli *et al*, 2003). Our results show that the colour polymorphism in *D. mediopunctata* is associated with chromosome inversions. Karyotype *PA0* was associated with phenotypes with fewer abdominal spots, whereas *PC0* was associated with phenotypes with more spots.

Owing to the strong linkage disequilibrium between proximal and distal inversions on the second chromosome, *PA0* was mainly linked to *DA*, whereas *PC0* was mainly linked to *DP*, *DV* and *DS* (Peixoto and Klaczko, 1991). Owing to this, the strains analysed in this work were *DA-PA0*, *DV-PC0*, *DS-PC0* and *DP-PC0*. Hence, it is possible that genes determining the colour polymorphism are also located in the distal region of this chromosome, and that the conclusions drawn here can be extended to the inversions in the distal region.

There was a significant interaction between karyotype and temperature, suggesting that the gene complexes within each karyotype had different reaction norms related to temperature.

Our results agree with two other data sets already found for this species. First, inversion frequencies in *D. mediopunctata* change during the year so that *PA0* tends to be more frequent in colder months than in warmer months, whereas *PC0* undergoes the opposite changes (Klaczko, 1995). These data suggest that *PA0* is more adapted to lower temperatures and *PC0* to higher temperatures. Second, we have circumstantial evidence (unpublished data) that, in natural populations, light genotypes increase in frequency with low temperatures, while dark genotypes decrease – although, due to plasticity, the average number of spots increases, that is, flies are phenotypically darker. Since *PA0* determines

phenotypes with fewer spots and this karyotype increases in frequency in colder months, an increase in the frequency of genotypes leading to phenotypes with fewer spots is also expected in colder months. Thus, a comprehensive picture – even if speculative – emerges for the patterns of variation in the inversions and the colour polymorphism. Since *PA0* seems to be adapted to lower temperatures, and *PC0* seems to be favoured by higher temperatures, it is possible that these inversions have undergone the accumulation of genes adapted for each of these temperature conditions. So, if the genes that determine abdominal colour patterns are located in these inversions, natural selection would favour an association in which the linked alleles would be more adapted to the temperatures in which each karyotype is the more frequent. That is, our results go exactly in the direction of the predictions of Ford (1964) that one should find an association of factors or genes determining a polymorphism forming a supergene, and that these genes would be kept together through a chromosome inversion, which would be the most efficient way to do it. This interpretation, however, remains speculative until further research confirms that the genes controlling the polymorphism are really within the inversions and that more than one gene is actually involved, hence representing a supergene. In addition, it is also necessary to investigate the adaptive significance of this colour polymorphism and whether it is under some type of balancing selection, thus helping to maintain the inversion polymorphism.

Moreover, it is necessary to study in more detail the finding of increased frequency of light genotypes with low temperatures in nature. This situation is the opposite of what is found for other species of *Drosophila*, which is commonly explained as an adaptive character related to the thermoregulation (the thermal budget hypothesis). For example, latitudinal clines in which genetically darker flies are found in higher latitudes were observed in the abdominal pigmentation of *D. melanogaster* (Das et al, 1994) and for the trident colouration of both *D. melanogaster* and *D. simulans* (Capy et al, 1988). Apparently, the variation in abdominal pigmentation in *D. mediopunctata* in nature can not be explained by the thermal budget hypothesis. This would lend some support for the ideas of Crill et al (1996). It opens the possibility of a number of alternative explanations, including linkage disequilibrium, which needs further investigation to be adequately tested.

We are presently working to answer these questions. Nevertheless, this is the first time an association between a conspicuous morphological polymorphism and chromosome inversions has been described.

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References

- Ananina G, Peixoto AA, Souza WN, Klaczko LB (2002). Polytene chromosome map and inversion polymorphism in *Drosophila mediopunctata*. *Mem Inst Oswaldo Cruz, Rio de Janeiro* **97**: 691–694.
- Bertrán E, Santos M, Ruiz A (1998). Antagonistic pleiotropic effect of second-chromosome inversions on body size and early life-history traits in *Drosophila buzzatii*. *Evolution* **52**: 144–154.
- Bitner-Mathé BC, Peixoto AA, Klaczko LB (1995). Morphological variation in a natural population of *Drosophila mediopunctata*: altitudinal cline, temporal changes and influence of chromosome inversions. *Heredity* **75**: 54–61.
- Brakefield PM (2003). The power of evo-devo to explore evolutionary constraints: experiments with butterfly eyespots. *Zoology* **106**: 283–290.
- Brakefield PM, Reitsma N (1991). Phenotypic plasticity, seasonal climate and the population biology of *Bicyclus* butterflies (Satyridae) in Malawi. *Ecol. Entomol.* **16**: 291–303.
- Calboli FCF, Kennington WJ, Partridge L (2003). QTL mapping reveals a striking coincidence in the positions of genomic regions associated with adaptive variation in body size in parallel clines of *Drosophila melanogaster* on different continents. *Evolution* **57**: 2653–2658.
- Capy P, David JR, Robertson A (1988). Thoracic trident pigmentation in natural populations of *Drosophila simulans*: a comparison with *D. melanogaster*. *Heredity* **61**: 263–268.
- Carvalho AB, Klaczko LB (1993). Autosomal suppressors of sex-ratio in *Drosophila mediopunctata*. *Heredity* **71**: 546–551.
- Crill WD, Huey RB, Gilchrist GW (1996). Within- and between-generation effects of temperature on the morphology and physiology of *Drosophila melanogaster*. *Evolution* **50**: 1205–1218.
- da Cunha AB (1949). Genetic analysis of the polymorphism of color pattern in *Drosophila polymorpha*. *Evolution* **3**: 239–251.
- Dahlgård J, Hasson E, Loeschcke V (2001). Behavioral differentiation in oviposition activity in *Drosophila buzzatii* from highland and lowland populations in Argentina: plasticity or thermal adaptation? *Evolution* **55**: 738–747.
- Das A, Mohanty S, Parida BB (1994). Inversion polymorphism and extra bristles in Indian populations of *Drosophila ananassae*: joint variation. *Heredity* **73**: 405–409.
- Dobzhansky T (1936). Studies on hybrid sterility. II. Localization of sterility factors in *D. pseudoobscura* hybrids. *Genetics* **21**: 113–135.
- Dobzhansky T (1970). *Genetics of the Evolutionary Process*, Columbia University Press: New York.
- Ford EB (1964). *Ecological Genetics*, Chapman & Hall: London.
- Freire-Maia N (1964). Segregational load in *Drosophila kikkawai* I. Crossing experiments. *Genetics* **50**: 211–219.
- Frota-Pessoa O (1954). Revision of the *tripunctata* group of *Drosophila* with description of fifteen new species (Drosophilidae, Diptera). *Arq Mus Para* **10**: 253–304.
- Gibert P, Moreteau B, Moreteau J, David JR (1996). Growth temperature and adult convergence of reaction norms in sympatric populations? *Evolution* **50**: 2346–2353.
- Gibert P, Moreteau B, Munjal A, David JR (1999). Phenotypic plasticity of abdominal pigmentation in *Drosophila kikkawai*: multiple interactions between a major gene, sex, abdomen segment and growth temperature. *Genetica* **105**: 165–176.
- Gordon IJ, Smith DAS (1998). Body size and colour-pattern genetics in the polymorphic mimetic butterfly *Hypolimnais misippus* (L.). *Heredity* **80**: 62–69.
- Heed WB, Blake PR (1963). A new color allele at the *e* locus of *Drosophila polymorpha* from northern South America. *Genetics* **48**: 217–234.
- Hollocher H, Hatcher JL, Dyreson EG (2000). Genetic and developmental analysis of abdominal pigmentation differences across species in the *Drosophila dunni* subgroup. *Evolution* **54**: 2057–2071.

- Izquierdo JI, García-Vázquez E, Villar B (1991). Correlated variation of chromosomal inversion (3R)C and extra bristles in *Drosophila melanogaster*. *Heredity* **67**: 183–187.
- Jones JS, Leith BH, Rawlings P (1977). Polymorphism in *Cepaea*: a problem with too many solutions? *Annu Rev Ecol Syst* **8**: 109–143.
- Klaczko LB (1995). Population genetics of *Drosophila medipunctata*. In: Levine L (ed) *Genetics of Natural Populations: The Continuing Importance of Theodosius Dobzhansky*, Columbia University Press: New York. pp 140–153.
- Kopp A, Duncan I, Carroll SB (2000). Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* **408**: 553–559.
- Lopart A, Elwyn S, Coyne JA (2002a). Pigmentation and mate choice in *Drosophila*. *Nature* **419**: 360.
- Lopart A, Elwyn S, Lachaise D, Coyne JA (2002b). Genetics of a difference in pigmentation between *Drosophila yakuba* and *Drosophila santomea*. *Evolution* **56**: 2262–2277.
- Mallet J, Barton N, Lamas G, Santisteban C, Muedas M, Eeley H (1990). Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. *Genetics* **124**: 921–986.
- Marples NM, DeJong PW, Ottenheim MM, Verhoog MD, Brakefield PM (1993). The inheritance of a wingless character in the 2-spot ladybird (*Adalia bipunctata*). *Entomol Exp Appl* **69**: 69–73.
- Martinez MN, Cordeiro AR (1970). Modifiers of color pattern genes in *Drosophila polymorpha*. *Genetics* **4**: 573–587.
- Murray J, Clarke B (1976). Supergenes in polymorphic land snails. I. *Partula taeniata*. *Heredity* **37**: 271–282.
- Noor MAF, Grams KL, Bertucci LA, Reiland J (2001). Chromosomal inversions and the reproductive isolation of species. *Proc Natl Acad Sci USA* **98**: 12084–12088.
- Peixoto AA, Klaczko LB (1991). Linkage disequilibrium analysis of chromosomal inversion polymorphisms of *Drosophila*. *Genetics* **129**: 773–777.
- Pipkin SB (1962). Mesonotal color polymorphism in *Drosophila lebanonensis*. *Genetics* **47**: 1275–1290.
- Powell JR (1997). *Progress and Prospects in Evolutionary Biology. The Drosophila Model*, Oxford University Press: New York.
- Rieseberg LH (2001). Chromosomal rearrangements and speciation. *Trends Ecol Evol* **16**: 351–358.
- Rodríguez C, Fanara JJ, Hasson E (1999). Inversion polymorphism, longevity and body size in a natural population of *Drosophila buzzatii*. *Evolution* **53**: 612–620.
- Saavedra CCR, Callegari-Jacques SM, Napp M, Valente VLS (1995). A descriptive and analytical study of our neotropical drosophilid communities. *J Zool Syst Evol Res* **33**: 62–74.
- Sokal RR, Rohlf FJ (1995). *Biometry: The Principles and Practice of Statistics in Biological Research*, WH Freeman and Co.: New York.
- Spicer GS (1991). The genetics basis of a species-specific character in the *Drosophila virilis* species group. *Genetics* **128**: 331–337.
- Stern D (2000). The problem of variation. *Nature* **408**: 529–530.
- Stevenson RD (1985). Body size and limits to the daily range of body temperature in terrestrial ectotherms. *Am Nat* **125**: 102–117.
- Vilela CR (1992). On the *Drosophila tripunctata* species group. *Rev Bras Ent mol* **36**: 197–221.
- Weeks AR, McKechnie SW, Hoffmann AA (2002). Dissecting adaptive clinal variation: markers, inversions and size/stress associations in *Drosophila melanogaster* from a central field population. *Ecol Lett* **5**: 756–763.
- Wijngaarden PJ, Brakefield PM (2000). The genetic basis of eyespot size in the butterfly *Bicyclus anynana*: an analysis of line crosses. *Heredity* **85**: 471–479.
- Wittkopp PJ, Williams BL, Selegue JE, Carroll SB (2003). *Drosophila* pigmentation evolution: divergent genotypes underlying convergent phenotypes. *Proc Natl Acad Sci USA* **100**: 1803–1813.
- Wright S, Dobzhansky T (1946). Genetics of natural populations. XII. Experimental reproduction of some of the changes caused by natural selection in certain populations of *Drosophila pseudoobscura*. *Genetics* **31**: 125–156.