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Manifestation of the Dilute Black (*bd*) Mutation and Constitution of the *bd* Locus in *Bombyx mori*

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A *Bombyx mori* mutant named dilute black (*bd*), which has the multiple alleles *bd*, bd^{sw} and bd^{f}, each affecting both the larval body color and female fertility, was investigated for the manifestation of these pleiotropic traits by mating experiments with the k40, k42 and k452 strains harboring *bd*, bd^{sw} and bd^{f}, respectively, in the heterozygous state. It was shown that *bd*, bd^{sw} and bd^{f} were strong, intermediate and weak, respectively, in the degree of larval body color. On the other hand, *bd* and bd^{sw} were identical to each other in terms of female sterility, whereas bd^{f} was as fertile as normal. The degree of body color and the female sterility showed a high parallelism with each other. On the basis of these results, we proposed that the *bd* locus consists of closely linked two domains, one for the larval body color and the other for the female fertility.

Additional Key word: Domesticated silkworm, Multiple alleles, Pleiotropic effects, Dual constitution of *bd* locus

INTRODUCTION

In the domesticated silkworm, *Bombyx mori*, many mutations related to the larval body color have previously been reported (Aruga *et al.*, 1951; Chikushi, 1972; Tazima *et al.*, 1975; Doira, 1986; Goldsmith, 1995; Fujii *et al.*, 1998). These include moricaud (*pM*), black (*pB*), sable (*pSa*), dirty (*Di*), sepialumadine (*Sel*), ursa (*U*), yellow molting (*Ym*) and xanthous (*Xan*) as dominant mutations, and albino (*al*), lemon (*lem*), dilute black (*bd*) and sooty (*so*) as recessive mutations. The gene controlling the dilute black (*bd*) trait was discovered as a spontaneous mutation belonging to the ninth linkage group, and was located at the map position of 22.9 (Doira *et al.*, 1992). This locus has multiple alleles named dilute black (*bd*), Chimney sweep (bd^{sw}) and dilute black fertile (bd^{f}) (Sasaki, 1941; Chikushi *et al.*, 1972; Shimizu and Matsuno, 1978). These *bd* alleles each express the traits of body color at the larval stage and female sterility at the adult stage, thus exhibiting pleiotropic effects (Sasaki, 1941; Tamura and Sakate, 1983). It was recently clarified that the sterility of *bd* and bd^{sw} females was caused by abnormality of the micropylar apparatus in the eggs (Kawaguchi *et al.*, 2006).

Here, we investigated, by mating experiments, the manifestation of the body color and female fertility due to the three alleles *bd*, bd^{sw} and bd^{f}. The degree of larval body color was confirmed to be in the decreasing order of *bd*, bd^{sw} and bd^{f}. Female sterility was marked in *bd* and bd^{sw} but not observed in bd^{f}, which was almost normal in fertility. There was a parallelism between the degree of larval body color and the adult female sterility. On the basis of these results, we advocated a scheme about the constitution of *bd* locus; it may comprise closely linked two domains, one for the larval body color and the other for the female fertility.

MATERIALS AND METHODS

Insects

The silkworm strains used were p22, k402, k422 and k452 maintained in the Faculty of Agriculture, Kyushu University Graduate School (Fujii *et al.*, 1998). The strain p22 is a standard Japanese race, bivoltine tetramolter, exhibiting normal body color at the larval stage and producing normal eggs after emergence. The k402, k422 and k452 strains possess the *bd*, bd^{sw} and bd^{f} gene, respectively, in the heterozygous state. These three strains had each received the W–translocated yellow blood gene to discriminate sex (cf. Kawaguchi *et al.*, 2006). Females of *bd* and bd^{sw} homozygotes are sterile, while females of bd^{f} homozygous are fertile. Mating between $+/bd$ females and *bd*/*bd* males, between $+/bd^{sw}$ females and bd^{sw}/bd^{sw} males or between $+/bd^{f}$ females and bd^{f}/bd^{f} males produces, in the same batch of offspring, a mixture of *bd*, bd^{sw} or bd^{f} homozygotes having the mutant body color (black arrows in Fig. 1A, B and C, respectively) and *bd*, bd^{sw} or bd^{f} heterozygotes having the normal body color (white arrows in Fig. 1A, B and C, respectively). Larvae were raised on mulberry leaves.

Mating manner and observation of traits

Mating experiment I was carried out to produce F_1,

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Fig. 1. Photographs of the fifth instar F_1 larvae from the crossings within the k402 strain having *bd* (A), the k422 strain having bd^{sw} (B) or the k452 strain having bd^{f} (C). Black allows indicate homozygous individuals with mutant body color (*bd/bd*, bd^{sw}/bd^{sw} and bd^{f}/bd^{f}, in A, B and C, respectively). White allows show coexisting heterozygotes with normal body color. The color degree of homozygotes was strong, intermediate and weak in A, B and C, respectively (cf. Kawaguchi *et al.*, 2006).

F_2 and BF_1 progeny by mating +/+ (p22) females with *bd/bd* males, bd^{sw}/bd^{sw} males or bd^{f}/bd^{f} males. Mating experiment II was done to get F_1 and F_2 offspring by mating bd^{f}/bd^{f} females with *bd/bd* males or bd^{sw}/bd^{sw} males. Mating experiment III was performed to obtain F_1 generation by reciprocal crossing between +/*bd* and +/bd^{sw}. Both sexes of larvae were observed for haemolymph (or blood) color. These experiments repeated 3 times. The degree of dilute black body color depended on the allele: strong, intermediate or weak (see the individuals pointed by black arrows in Fig. 1A, B and C, respectively). Animals were then raised until the adult stage and subjected to mating. Female moths were allowed to deposit eggs, whose fertilization rate was >80% or 0% when determined as described previously (Kawaguchi *et al.*, 2006), and assigned as fertile or sterile, respectively. All results about the body color and sterility obtained in this investigation were simply expressed in the segregating ratios.

RESULTS

Segregation of traits after crossing between normal and each homozygote (mating experiment I)

The manifestation of the traits of larval body color and female fertility due to the *bd*, bd^{sw} and bd^{f} alleles was investigated by observing the F_1, F_2 and BF_1 progeny from the mating experiment I performed in three crossings: I–1, +/+ females × *bd/bd* males; I–2, +/+ females × bd^{sw}/bd^{sw} males; I–3, +/+ females × bd^{f}/bd^{f} males. Results are summarized in Table 1.

All F_1 individuals from these crossings were normal both in body color of larvae and in fertility of female adults. The F_2 and BF_1 larvae from all crossings segregated with respect to the body color into normal and mutant (dilute black) at ratios of 3 : 1 and 1 : 1, respectively. As written in footnotes of the table, the mutant body color was strong, intermediate or weak, and these characteristics must correspond to *bd/bd*, bd^{sw}/bd^{sw} and bd^{f}/bd^{f}, respectively (cf. Fig. 1). The F_2 and BF_1 female

Table 1. Segregation ratios in F_1, F_2 and BF_1 from the crossings of normal with different *bd* homozygotes

I–1. Normal (+/+) ♀ × *bd/bd* ♂

Larval body color	Normal		Dilute black *	
Fertility	Fertile	Sterile	Fertile	Sterile
F_1	1	0	0	0
F_2	3	0	0	1
B F_1	1	0	0	1

* The degree of larval body color was strong in all.

I–2. Normal (+/+) ♀ × bd^{sw}/bd^{sw} ♂

Larval body color	Normal		Dilute black **	
Fertility	Fertile	Sterile	Fertile	Sterile
F_1	1	0	0	0
F_2	3	0	0	1
B F_1	1	0	0	1

** The degree of larval body color was intermediate in all.

I–3. Normal (+/+) ♀ × bd^{f}/bd^{f} ♂

Larval body color	Normal		Dilute black ***	
Fertility	Fertile	Sterile	Fertile	Sterile
F_1	1	0	0	0
F_2	3	0	1	0
B F_1	1	0	1	0

*** The degree of larval body color was weak in all.

adults from the crossings I–1 and I–2 segregated into fertile and sterile at ratios of 3 : 1 and 1 : 1, respectively. Note that, in the crossings I–1 and I–2, all individuals with normal body color were fertile, while those with mutant body color were sterile. On the other hand, the F_2 and BF_1 progeny of the crossing I–3 did not show sterility; i.e., all females were fertile despite that their larval body color was normal or not.

Segregation of traits after crossing between homozygotes (mating experiment II)

Characteristics of progeny were investigated after mating experiment II, which included two crossings: II–1, bd^f/bd^f females × bd/bd males; II–2, bd^f/bd^f females × bd^{sw}/bd^{sw} males. Results are summarized in Table 2.

Table 2. Segregation ratios of F_1 and F_2 in the crossings between different *bd* homozygotes

II–1. bd^f/bd^f ♀ × bd/bd ♂

Larval body color	Normal		Dilute black	
Fertility	Fertile	Sterile	Fertile	Sterile
F_1	0	0	1[a]	0
F_2	0	0	3[b]	1[c]

[a] The degree of larval body color was intermediate in all.
[b] The degree of larval body color was 2 intermediate : 1 weak.
[c] The degree of larval body color was strong in all.

II–2. bd^f/bd^f ♀ × bd^{sw}/bd^{sw} ♂

Larval body color	Normal		Dilute black	
Fertility	Fertile	Sterile	Fertile	Sterile
F_1	0	0	1[d]	0
F_2	0	0	3[e]	1[f]

[d] The degree of larval body color was intermediate in all.
[e] The degree of larval body color could not be distinguished.
[f] The degree of larval body color was intermediate in all.

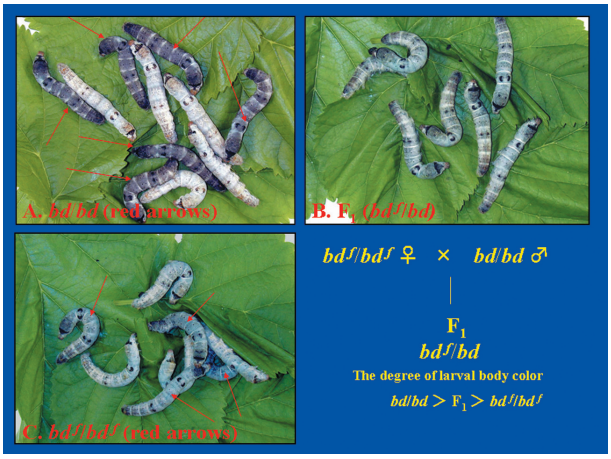

Fig. 2. Photographs of the fifth instar larvae with genotypes of *bd/bd* (A), bd/bd^f (B) and bd^f/bd^f (C). In panels A and C, homozygotes with mutant body color (dilute black) are discriminated by red allows from coexisting heterozygotes. Larvae shown in panel B are F_1 from the crossing of *bd/bd* females and bd^f/bd^f males (scheme in the bottom–right panel; cf. also II–1 of Table 2). The color degree of dilute black was strong, intermediate and weak in A, B and C, respectively.

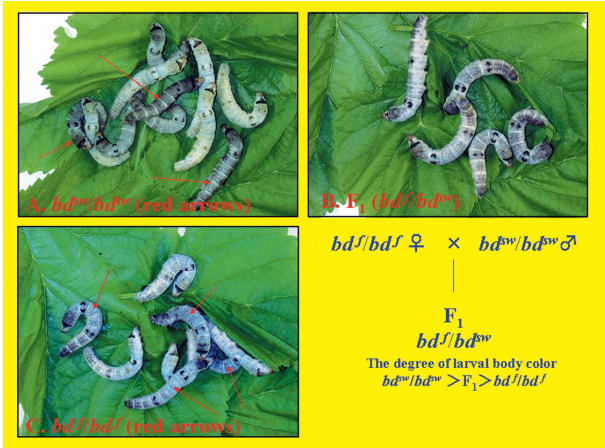

Fig. 3. Photographs of the fifth instar larvae with genotypes of bd^{sw}/bd^{sw} (A), bd^f/bd^{sw} (B) and bd^f/bd^f (C). In panels A and C, homozygotes with mutant body color (dilute black) are discriminated by red allows from coexisting heterozygotes. Larvae shown in panel B are F_1 from the crossing of bd^f/bbd^f females and bd^{sw}/bd^{sw} males (scheme in the bottom–right panel; cf. also II–2 of Table 2). The color degree of dilute black was in the decreasing order: A>B>C.

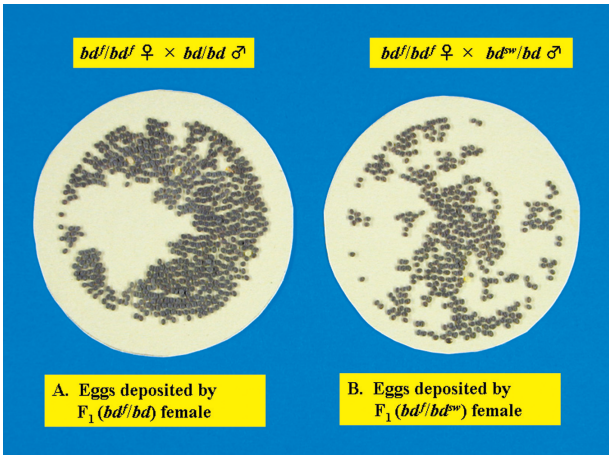

Fig. 4. Photographs of eggs deposited by F_1 females from the different crossings. A, bd^f/bd^f females × bd/bd males; B, bd^f/bd^f females × bd^{sw}/bd^{sw} males.

In the case of II–1, the F_1 larvae (bd^f/bd) were all dilute black in larval body color, whose degree was intermediate (footnote a in the table). In F_2, again all larvae had mutant body color, but segregating into strong, intermediate and weak at a ratio of 1 : 2 : 1 (footnotes b and c in the table). Thus, we concluded that the degree of larval body color varied in the order of $bd/bd > F_1 > bd^f/bd^f$ (relevant larvae are shown in Fig. 2A, B and C, respectively). All F_1 female adults were normal in fertility (deposited eggs are illustrated in Fig. 4A). The F_2 female adults (all with mutant body color at the larval stage) exhibited a 3 : 1 fertile to sterile ratio. As a whole, the individuals whose larval body color was strong were sterile, while those whose larval body color was intermediate or weak were fertile.

In the case of II–2, the F_1 and F_2 individuals showed the same segregation ratios as in II–1 with respect to

both body color and fertility. Parent and F_1 specimens at the larval stage are seen in Fig. 3A, B and C, and eggs deposited by F_1 in Fig. 4B. The degree of body color was again intermediate in F_1 (footnote d in the table). All F_2 population had the mutant body color, whose segregation into different degrees was equivocal, although the individuals with intermediate body color showed female sterility (footnotes e and f in the table).

Segregation of traits after crossing between heterozygotes (mating experiment Ⅲ)

Mating experiment Ⅲ was conducted by reciprocal crossing between $+/bd$ and $+/bd^{sw}$ with schemes denoted as Ⅲ–1 and Ⅲ–2 (Table 3). Both types of progeny segregated with respect to larval body color into normal and mutant at a ratio of 3 : 1. As written in footnotes of the table, the degree of dilute black color was strong. The female adults whose larval color was normal were fertile, while those with mutant body color were sterile.

Table 3. Segregation ratios of progeny in the crossings between different *bd* heterozygotes

Ⅲ–1. $+/bd$ ♀ × $+/bd^{sw}$ ♂

Larval body color	Normal		Dilute black *	
Fertility	Fertile	Sterile	Fertile	Sterile
Progeny	3	0	0	1

* The degree of larval body color was strong in all.

Ⅲ–2. $+/bd^{sw}$ ♀ × $+/bd$ ♂

Larval body color	Normal		Dilute black *	
Fertility	Fertile	Sterile	Fertile	Sterile
Progeny	3	0	0	1

* The degree of larval body color was strong in all.

DISCUSSION

Since the discovery of the *bd* mutation by Sasaki (1941), the locus of relevant gene was once located at the map position of 6.7 on the ninth linkage group. Afterwards, the *bd* locus was changed to the present position of 22.9 (Doira *et al.*, 1992) on the basis of the genetic analysis of the Japanese translucent (*oj*) gene, which had been discovered by Doira (1989). Because it was identified that the *oj* gene belonged to the ninth linkage group and was located at the left of *I* gene (9–0.0), the genetic map of the ninth linkage group was revised as *oj* (0.0), *I* (16.2), *nm–d* (16.3), *gn* (22.0), *Ia* (22.1), *bd* (22.9), *og* (23.6) and *l–br* (34.3).

The *bd* locus, having at least three alleles, *bd*, bd^{sw} and bd^{f}, brings about two distinct and seemingly unrelated phenotypic effects (pleiotropism) on the larval body color and the adult female fertility (although bd^{f}/bd^{f} females are fertile). Results obtained by the present mating experiments indicated that the order of relative intensity of the allelic traits was $bd > bd^{sw} > bd^{f}$ with respect to the larval body color, and bd $bd^{sw} >$ bd^{f} with respect to the adult female sterility. The manifestation of larval body color and that of female sterility kept a clear parallelism with each other. We assumed that the *bd* locus consists of two closely adjoined parts, the larval body color domain and the female fertility domain, and tried to propose the constitution of *bd* locus in the ninth chromosome (schematically shown in Fig. 5), by which the results of mating experiments Ⅰ, Ⅱ and Ⅲ (processes detailed in MATERIASLS AND METHODS) could be well explained. Some differences in base sequence and/or length at the body color domain among the alleles may make the body color strong, intermediate or weak. Structure at the fertility domain may be similarly disordered in the *bd* and bd^{sw} alleles but almost normal in the

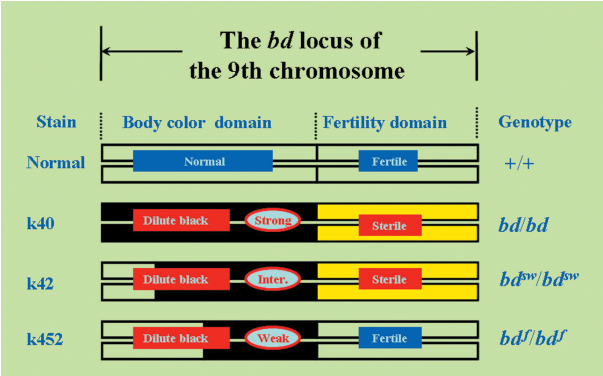

Fig. 5. Schematic representation showing the dual constitution of *bd* locus in the ninth chromosome of *B. mori*. It was assumed that the *bd* locus consists of the larval body color domain and the female fertility domain in order to explain the results presented in this article, in which mating experiments were performed with strains harboring *bd*, bd^{sw} and bd^{f} alleles (k402, k422 and k452, respectively). Homozygous alleles are represented by paired boxes. Some differences in base sequence and/or length at the body color domain may control the degree of mutant body color, strong, intermediate or weak. Structures may be highly similar between the fertility domain of *bd* and bd^{sw}, as well as between + and bd^{f}.

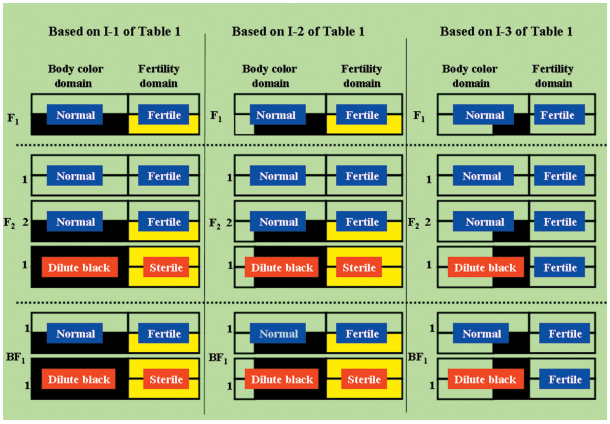

Fig. 6. Putative constitution of *bd* locus in F_1, F_2 and BF_1 from the crossings I–1, I–2 and I–3 of Table 1. Homozygous or heterozygous alleles are represented by paired boxes. Numerical frequency ratios of genotypes are drawn besides the boxes. See Fig. 5 for other explanations about the proposed organization.

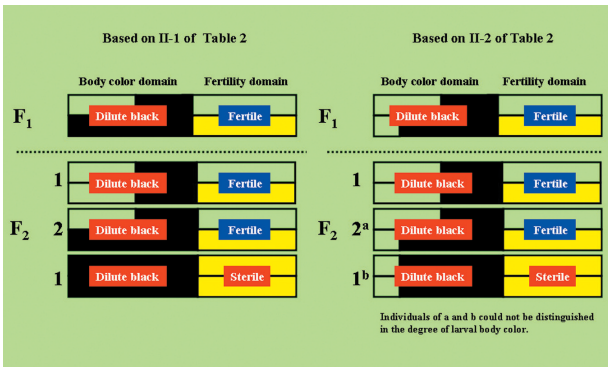

Fig. 7. Putative constitution of *bd* locus in F_1 and F_2 from the crossings II–1 and II–2 of Table 2. See the legend to Fig. 6 for other details.

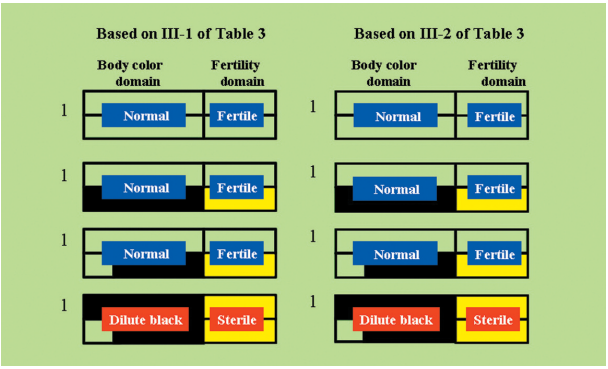

Fig. 8. Putative constitution of *bd* locus in progeny from the reciprocal crossing III–1 and III–2 of Table 3. See the legend to Fig. 6 for other details

bd^f allele. The segregation status of *bd* alleles in progeny given in Tables 1 and 2 could be mirrored in displays shown in Figs. 6 and 7, respectively, which supported the idea that the *bd* locus has dual constitution. Only two phenotypes are seen in the progeny given in Table 3, i.e., normal/fertile and dilute–black/sterile, but four genotypes exist as illustrated in Fig. 8.

We concluded that the present postulation for the two–domain constitution of *bd* locus, as shown in Fig. 5, is valid to explain the results of the current and previous mating experiments performed by applying the three *bd* strains. It is expected that the present assumption is confirmed by molecular level analyses if the relevant sequences could be found in the huge volumes of DNA database compilation updated in recent years.

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