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yellow and *ebony* Are the Responsible Genes for the Larval Color Mutants of the Silkworm *Bombyx mori*

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ABSTRACT

Many larval color mutants have been obtained in the silkworm *Bombyx mori*. Mapping of melanin-synthesis genes on the Bombyx linkage map revealed that *yellow* and *ebony* genes were located near the *chocolate* (*ch*) and *sooty* (*so*) loci, respectively. In the *ch* mutants, body color of neonate larvae and the body markings of elder instar larvae are reddish brown instead of normal black. Mutations at the *so* locus produce smoky larvae and black pupae. F_2 linkage analyses showed that sequence polymorphisms of *yellow* and *ebony* genes perfectly cosegregated with the *ch* and *so* mutant phenotypes, respectively. Both *yellow* and *ebony* were expressed in the epidermis during the molting period when cuticular pigmentation occurred. The spatial expression pattern of *yellow* transcripts coincided with the larval black markings. In the *ch* mutants, nonsense mutations of the *yellow* gene were detected, whereas large deletions of the *ebony* ORF were detected in the *so* mutants. These results indicate that *yellow* and *ebony* are the responsible genes for the *ch* and *so* loci, respectively. Our findings suggest that Yellow promotes melanization, whereas Ebony inhibits melanization in Lepidoptera and that melanin-synthesis enzymes play a critical role in the lepidopteran larval color pattern.

THE extremely diverse lepidopteran color pattern is evolutionarily interesting because of its association with natural selection. Much research has focused on adult wings to study the molecular mechanisms of color patterns. Some of the most convincing data comes from comparative studies between different species (Carroll *et al.* 1994; Brunetti *et al.* 2001; Reed and Serfas 2004; Monteiro *et al.* 2006), phenotypically differentiated laboratory strains, or spontaneous mutants within species (Brakefield *et al.* 1996; Brunetti *et al.* 2001, Beldade *et al.* 2002). A candidate gene approach revealed that the *Distal-less* gene segregates with the eyespot size phenotype, explaining up to 20% of the phenotypic difference between the selected lines in *Bicyclus anynana* (Beldade *et al.* 2002). To determine the responsible genes for color pattern polymorphisms or mutants, an AFLP-based linkage map has been developed in several butterfly species (reviewed in Beldade *et al.* 2008). Recently, the linkage of forewing color pattern and mate preference with the *wingless* gene in two Heliconius species (Kronforst *et al.* 2006) and the linkage of the mimicry locus H with the *invected* gene in *Papilio dardanus* have been reported (Clark *et al.* 2008), although these reports have not elucidated whether *wingless* or *invected* is the responsible gene for wing color pattern variation. Until now, no color pattern genes have been elucidated by positional cloning in Lepidoptera.

Like the adult wings, the larvae of butterflies and moths, often preyed on by other animals, also show various color patterns. In the swallowtail butterfly, *Papilio xuthus*, several melanin-synthesis genes are associated with stage-specific larval color patterns (Futahashi and Fujiwara 2005, 2006, 2007, 2008a). Melanin-synthesis genes are responsible for pigmentation mutants in *Drosophila melanogaster* (Wright 1987; Wittkopp *et al.* 2002a); however, the connection between these genes and the color pattern mutants in other insects has not been elucidated.

Although larval color variations are often observed in many Lepidoptera, the genes responsible for color patterns have not yet been identified by mutation studies.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AB438999–AB439000, AB439286–AB439288, and AB455226–AB455233.

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Elucidating the genetic basis of lab-generated color mutants is important because it points out the interacting loci in the pathway that produces interesting phenotypes (larval pigmentation in this case) and it highlights genetic changes that could serve as the raw material for evolutionary change. Among lepidopteran species, the silkworm *Bombyx mori* is the most suitable for identification of mutants because its genome is already available (Mita *et al.* 2004; Xia *et al.* 2004); a high-density linkage map has been constructed between p50T and C108T strains (Yamamoto *et al.* 2006, 2008); and many available color mutants, especially in larval stages, have been obtained (Banno *et al.* 2005). Here we have analyzed whether melanin-synthesis genes were associated with Bombyx larval color mutants by using linkage analysis and comparing protein structure between wild-type and mutant strains. These genes are predicted to be important for driving patterns of pigmentation that may be used as a mechanism to avoid being preyed upon. Linkage analysis revealed perfect cosegregation between the *chocolate* (*ch*) locus and the *yellow* gene and between the *sooty* (*so*) locus and the *ebony* gene. The spontaneous *ch* mutant was first reported in Toyama (1909) and was mapped at 9.6 cM of the silkworm genetic linkage group 13 (Suzuki 1942; Banno *et al.* 2005). In the recessive homozygote of the *ch* mutant, the larval skin and the head cuticle of newly hatched larvae is reddish brown instead of the normal black (Figure 1B). In grown larvae of the homozygous *ch* mutants, black body markings and sieve plates of spiracles remain reddish brown (Figure 1A). The *so* is also a spontaneous mutant (Tanaka 1924) and was mapped at the end of the silkworm genetic linkage group 26 (Banno *et al.* 1989, 2005). In the recessive homozygote of the *so* mutant, the pupal color is black, especially at the ventral tip of the abdomen (Figure 1C). From larvae to the adult stage, body color is smoky, but less conspicuous compared to pupae (Figure 1A). Molecular characterization of these pigmentation mutants demonstrated that *ch* mutants were loss-of-function *yellow* alleles caused by a deletion or a presumptive splice junction mutation, while the *so* mutants were loss-of-function *ebony* alleles caused by deletions present in 3′ exons, suggesting that these two genes were responsible for the black color pattern common among insects.

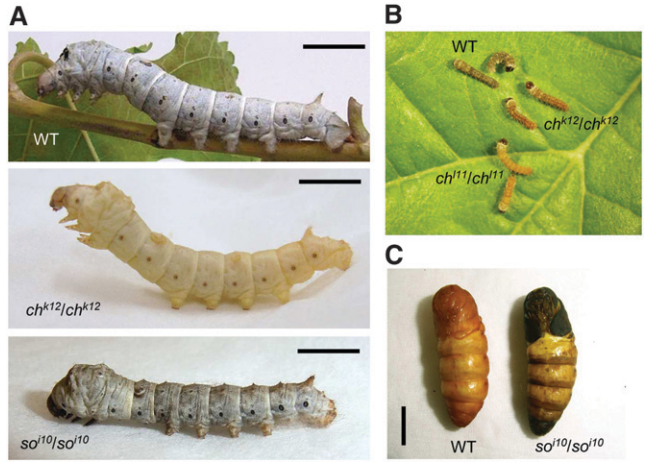

Figure 1.—(A) Lateral view of the fifth instar larva of silkworm *B. mori*. Wild type ($+^{p}$ strain, top), *chocolate* mutant (ch^{k12}, middle), and *sooty* mutant (so^{i10}, bottom) are shown. Bars, 1 cm. (B) The *B. mori* neonate larvae of wild-type (p50T strain) and *chocolate* mutants (ch^{l11} and ch^{k12}). The body color of wild type (WT) is brownish black while that of *ch* mutants is reddish. (C) Pupal black phenotype of *sooty* mutant (right). Bar, 1 cm.

MATERIALS AND METHODS

Silkworm strains: The ch^{k12}, ch^{l11}, so^{i10}, and so^{i41} mutant strains were provided from the silkworm stock center of Kyushu University supported by the National BioResource Project. The silkworms were reared with mulberry leaves or artificial diets (Nihon Nosan Kogyo, Yokohama, Japan) under a 16-hr-light:8-hr-dark photoperiod at 25°. The staging of the molting period was based on the spiracle index, which represented the characteristic sequence of new spiracle formation (Kiguchi and Agui 1981).

Mapping and linkage analysis: For the linkage map construction, we have developed web-based in-house software designed to assist positional cloning in silkworm genomic research. This is a Perl-based Common Gateway Interface program. This program requires BioPerl (Stajich *et al.* 2002), BLAST (Altschul *et al.* 1997), primer3 (Rozen and Skaletsky 2000), and RepeatMasker (http://www.repeatmasker.org/). After checking the validity of the query sequence, BLAST search of the query sequence against the silkworm genomic sequence database was performed (Mita *et al.* 2004; Xia *et al.* 2004). The repeat sequence masking of the sequence was internally performed with RepeatMasker previous to primer design with primer3. Single-nucleotide polymorphism (SNP), including small base insertions and deletions, were identified using the above primers. We designed two primer sets for each gene and used the following polymerase chain reaction (PCR) primer pairs: 5′-TGATGCTACTGACTGACCTTGA-3′ and 5′-TAACTTGATGCAAATGGTATTTTT-3′ for *TH*, 5′-AGTCCGCTATCTTGTCTAATGAACTAGGTG-3′ and 5′-ATAACTCCGTTTCTGTATGAAAGAAGGACA-3′ for *DDC*, 5′-GAGACGAAACTAAAGTGAAAGGTTCCTA-3′ and 5′-CAACAATCTTGTACCGACCTAGTACTTAT-3′ for *yellow*, 5′-CTAACAACTGCCATTCTCTTAGCATGATTT-3′ and 5′-TGCTCTTTCGAACAGAAAAATAGAACGTAT-3′ for *ebony* (SNP 1), and 5′-CAAGCTTAAACCTTCGAGGAGAACTACTTT-3′ and 5′-CGACACAGATTAACCTGAACAATGAATACT-3′ for *ebony* (SNP 2). Segregation patterns of SNPs were surveyed using 190 first-generation backcross (BC_1) individuals from a single pair mating between a p50T female and an F_1 male (p50T female × C108T male) as previously reported (Yamamoto *et al.* 2006, 2008). Segregation patterns were analyzed using Mapmaker/exp (version 3.0; Lander *et al.* 1987) with the Kosambi mapping function (Kosambi 1944).

The linkage analysis between the *ch* locus and *yellow* gene was estimated by SNP analysis. The cross that showed tight linkage between *yellow* and ch^{k12} was an F_2 intercross (heterozygous F_1 females mated to F_1 heterozygous males). Genomic DNA was extracted from the parent moths, F_1 moths, and F_2 larvae (first instar). A genomic fragment of the *yellow* gene was amplified by PCR with the following primers: 5′-CTCGTGTCGCAAGACGGATAGC-3′ and 5′-CCTTGTGTAGCGACCATGTCAC-3′. The linkage analysis between the *so* locus and *ebony*

gene was estimated on the basis of the length of the PCR fragment of the *ebony* gene. The cross that showed tight linkage between *ebony* and so^{i41} was a backcross (*so* mutant female mated to an F_1 heterozygous male). Genomic DNA was extracted from the parent moths, F_1 moths, and F_2 pupae. A genomic fragment of the *ebony* gene was amplified by PCR with the following primers: 5′-CGTGGTGCTATGCTACGGTT-3′ and 5′-TTGCCGTTTACCAGCAGAGG-3′.

Cloning of *yellow* and *ebony* cDNAs: Total RNA was isolated from several tissues by the TRI-reagent kit (Sigma, St. Louis) and reverse transcribed with random primer (N6) by the first-strand cDNA synthesis kit (Amersham, Sunnyvale, CA). The full-length cDNA was obtained by rapid amplification of cDNA ends (RACE) technique using the Marathon cDNA amplification kit (Clontech, Mountain View, CA). The following primers were used: 5′-GCTGGACACCGGAGTCGTCCATTAC-3′ for 5′ RACE of *yellow*, 5′-TGCCAACATCGCTCTCGATATCG-3′ and 5′-CCGATGAACTGGGCTATGGTCTTATC-3′ for 3′ RACE of *yellow*, 5′-ACGGGTCGGGCTCAACTCCTCATC-3′ and 5′-GGG TACAAATCCAATTGGTCGCTGCCT-3′ for 5′ RACE of *ebony*, and 5′-CGCACAGGAGATTTCGGGACTCTTG-3′ and 5′-GA CACCGCGTGGATCTGCTGGAAGT-3′ for 3′ RACE of *ebony*. PCR was performed using ExTaq (TaKaRa) under the following conditions: 35 cycles at 94° for 30 sec, 55° for 30 sec, and 72° for 90 sec. The PCR products were subcloned into pGEM-T Easy Vector (Promega, Madison, WI) and sequenced by an ABI3130xl genetic analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis: To investigate whether we obtained the genuine orthologs of melanin-synthesis genes in *B. mori*, we performed phylogenetic analysis using several related genes. The sequences used to create the diagram are listed in Table 1. Sequences were aligned using Clustal_X (Thompson *et al.* 1997). Phylogenetic trees were constructed by the neighbor-joining method with the MEGA4 program (Tamura *et al.* 2007). The confidence of the various phylogenetic lineages was assessed by the bootstrap analysis.

Reverse transcription–polymerase chain reaction analyses: Total RNA from several tissues (fat body, midgut, Malpighian tubules, epidermis, tracheae, and posterior silk gland) at day 3 of the fifth instar were extracted using TRI reagent (Sigma) and reverse transcribed with random primer (N6) and a first-strand cDNA synthesis kit (Amersham). The following primer sets were used for *yellow*: SO, 5′-TGAGTAAATAAAATGG CAGCGAAG-3′; S3, 5′-GAACAGAACAAGTCATGGAGATT-3′; ASO, 5′-TCTAGGAATTGAGAATTTGAACCA-3′; and AS2, 5′-GCGTTTTGGTCGATCAAGTTGAA-3′. And for *ebony*, the following primer sets were used: S4, 5′-TCCTCTGCT GGTAAACGGCA-3′; AS3, 5′-TCCAGCTCGGCTTTCTCGTA-3′; AS4, 5′-CGTGAACACGCCTCTGAAGC-3′; AS5, 5′-CGGA ACCCTCCACGTACTCC-3′; and AS6, 5′-TGGTGAGATTCTC GATCTCG-3′. The PCR conditions used were 96° for 2 min followed by 30 (or 33) cycles of 96° for 15 sec, 50° (or 52°) for 15 sec, and 72° for 1 min. The reactions were kept at 72° for 1 min after the final cycle. The gene for *Actin 3* was used as an internal control for normalization of equal sample loading.

Northern analysis: Total RNA (10 μg) was separated on a formaldehyde–agarose (1%) gel and transferred to a Hybond-N nylon membrane (Amersham). Hybridization was performed at 42° for 18 hr in 50% formamide, 5× SSC (1× SSC is 0.15 M sodium chloride and 0.15 M sodium citrate, pH 7.4), 10× Denhardt's solution (0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 25 μg/ml sonicated salmon sperm DNA, 50 mM sodium phosphate (pH 7.0), and ^{32}P-labeled DNA. Each DNA probe was labeled with [α-^{32}P]dCTP using a BcaBEST labeling kit (TaKaRa, Otsu, Shiga, Japan). DNA probes were synthesized by PCR using the following primers: 5′-TGGGCTAGTCTCACTAGCATCAGC-3′ and 5′-AGCGGATGAAGTTTGTTTCGG-3′ for *yellow* (to test the developmental profile in epidermis), 5′-GAAGG TATCTTCGGCATCACG-3′ and 5′-ATACCGCAACGGCTTCA GAG-3′ for *yellow* (to test tissue specificity), and 5′-ACCG GCATTCCGAAAGGTGTGCGTT-3′ and 5′-TCACAGAAACG GTTCGCGCC-3′ for *ebony*. The membranes were washed twice at room temperature for 20 min in 2× SSC with 0.1% sodium dodecyl sulfate (SDS). The further washes were followed by 30 min at 65° successively in 2× SSC with 0.1% SDS and in 0.2× SSC with 0.1% SDS.

Whole-mount *in situ* hybridization: Larval epidermis was dissected and then fixed immediately in 4% paraformaldehyde in phosphate-buffered saline (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, and 1.47 mM KH_2PO_4, pH 7.4). Whole-mount *in situ* hybridization was performed as described by Futahashi and Fujiwara (2005, 2008b). RNA probes for *yellow* and *ebony* were prepared using a digoxigenin (DIG) RNA labeling kit (Roche Biochemicals, Mannheim, Germany) and primers were as described above in *Northern analysis*. Digoxigenin-labeled antisense RNA probes were used, and the color reaction was performed at room temperature in 100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$ (pH 9.5) containing 3.5 μl/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt, and 4.5 μl/ml nitroblue tetrazolium chloride. Digoxigenin-labeled sense-strand probes were used as negative controls.

RESULTS

Mapping of the melanin-synthesis genes: We focused on the five melanin-synthesis (or related) genes, *tyrosine hydroxylase* (TH), *dopa decarboxylase* (DDC), *guanosine triphosphate cyclohydrolase I* (GTPCH I), *yellow*, and *ebony*, all of which are associated with stage-specific larval body markings in *Papilio xuthus* (Futahashi and Fujiwara 2005, 2006, 2007, 2008a). In *B. mori*, *DDC* (Hwang *et al.* 2003) has been reported in the full-length cDNA, and both *GTPCH I* (Kato *et al.* 2006) and *yellow* (Xia *et al.* 2006) have been reported in a partial sequence. We cloned melanin-synthesis genes using genomic information, reverse transcription–polymerase chain reaction (RT–PCR), and RACE. We obtained the full-length cDNA sequences of *B. mori TH* (GenBank accession no. AB439286), *yellow* (AB438999), and *ebony* (AB439000). We found two isoforms of the *GTPCH I* gene (AB439287 and AB439288), which encode proteins of distinct N termini as with *P. xuthus* (Futahashi and Fujiwara 2006). Phylogenetic analysis of these genes indicated that they cluster with other insect homologs and thus represent the *TH*, *GTPCH I*, *yellow*, and *ebony* orthologs in *B. mori* (Figure 2, Table 1). Although Yellow proteins form a large protein family (Albert and Klaudiny 2004), phylogenetic analysis indicated that *B. mori* Yellow clusters with other insect *yellow* homologs and thus represents the genuine *yellow* ortholog of *B. mori* (Figure 2). To determine the chromosomal location of the five melanin-synthesis (or related) genes, we performed genetic linkage analyses using 190 BC_1 individuals from a single pair mating between a p50T female and an F_1 male (p50T female × C108T male; Yamamoto *et al.* 2006, 2008). We constructed SNP markers of these genes (supplemental Figure 1), and the SNP markers segregated into 1 of 28 linkage groups as previously

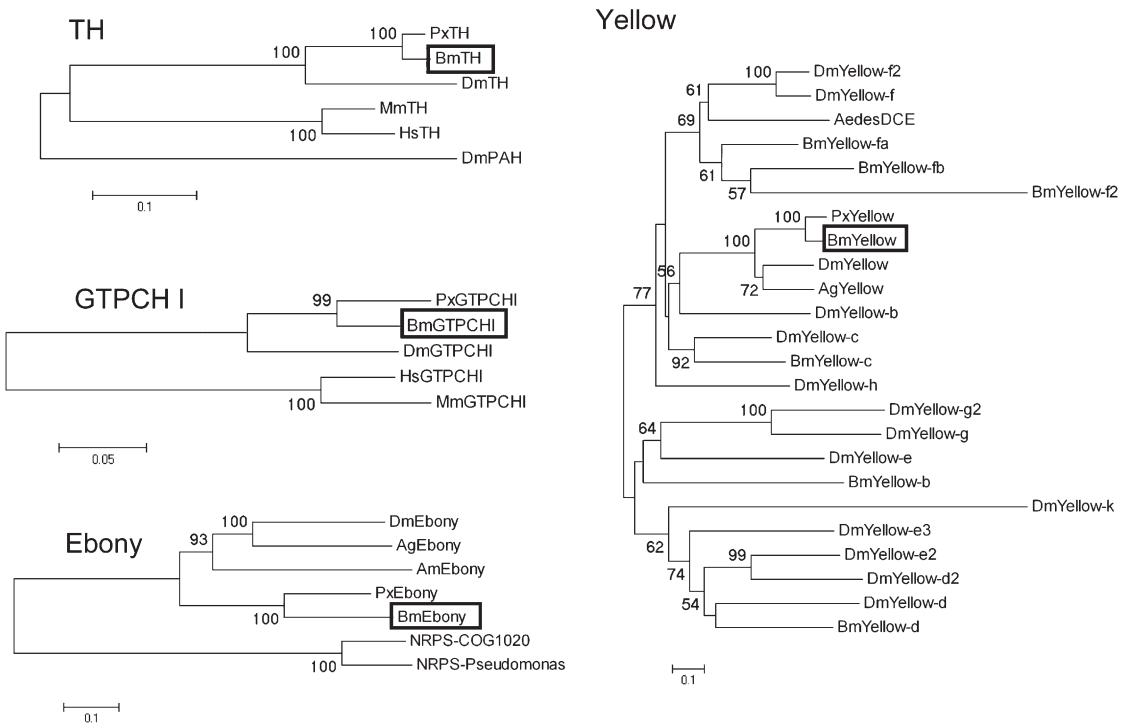

Figure 2.—Neighbor-joining tree of TH, GTPCH I, Yellow, Ebony, and related genes based on their amino acid sequences. The numbers at the tree edges represent the bootstrap values. The scale bars indicate the evolutionary distance between the groups. Boxes indicate the *B. mori* orthologs. The sequences used to create the diagram are listed in Table 1.

reported (Yamamoto *et al.* 2006, 2008). We could not obtain a clear result for the *GTPCH I* gene, perhaps because of the duplication of SNP markers. Except for *GTPCH I*, each of the four melanin-synthesis (or related) genes was located on a distinct linkage group (Figure 3, Table 2). Among them, *yellow* (27.9 cM of the SNP linkage group 13) and *ebony* (the end of the SNP linkage group 26) were located near the two color mutants, *ch* (9.6 cM of the phenotypic linkage group 13) and *so* (the end of the phenotypic linkage group 26), respectively (Figure 3, Table 2). Although the number of the SNP linkage group is the same as that of the phenotypic linkage group, the orientation of the SNP linkage group is not always the same as that of the phenotypic linkage group. We therefore analyzed the relationships by linkage analyses between the wild-type and mutant strains.

Linkage analysis of the *yellow* gene and the *ch* locus: To determine the linkage between the *ch* locus and the *yellow* gene, we performed an F_2 linkage analysis based on an SNP of the *yellow* gene. The first base of intron 3 of the *yellow* gene of the wild-type p50T strain was guanine, while that of the ch^{k12} strain was adenine (this substitution caused nonsense mutations; see below). The coloration of neonate larvae of F_2 individuals was either black or reddish brown (see Figure 1B). By analyzing 179 individuals obtained from an F_2 intercross (heterozygous F_1 females mated to F_1 heterozygous males), we found that all 93 reddish-brown specimens (ch^{k12}/ch^{k12}) were A/A homozygous, while all 86 black (+/+, $ch^{k12}/+$) F_2 larvae were G/G homozygous or A/G heterozygous (Table 3). These results indicate that the *yellow* gene is tightly linked to the *ch* locus (recombination value <0.56%). Because the 1-cM distance on the Bombyx classical genetic map is estimated to be ~300–600 kb (Sato *et al.* 2008), the region of linkage in the F_2 analysis is expected to be ~170–340 kb.

Linkage analysis of the *ebony* gene and the *so* locus: To determine the linkage between the *so* locus and the *ebony* gene, we performed an F_2 linkage analysis based on the length of *ebony* gene PCR fragments. The lengths of the amplified fragments of the two primers designed from the 10th intron of the *ebony* gene were 1.3 kb in wild-type p50T and 0.8 kb in mutant so^{i41}. The pupal coloration of F_2 individuals was either black (*so* mutant) or light brown (wild type; see Figure 1C). By analyzing 191 individuals obtained from a backcross (so^{i41} mutant female mated to an F_1 heterozygous male), we found that all 97 black pupae (so^{i41}/so^{i41}) had only 0.8-kb PCR fragments, while all 94 light-brown pupae ($so^{i41}/+$) had both 1.3- and 0.8-kb PCR fragments (Table 4). These results indicate that the *ebony* gene is tightly linked to the *so* locus (with the recombination value <0.52%, the region of linkage in the backcross analysis is expected to be ~150–300 kb.)

Expression analysis of *yellow* and *ebony* genes: In the swallowtail butterfly, *P. xuthus*, *yellow* expression coincided with larval black markings in the middle of the molting period (Futahashi and Fujiwara 2007), whereas *ebony* expression coincided with larval reddish-

TABLE 1

Abbreviation table of proteins used in the neighbor-joining tree in Figure 2

Abbreviation	Species	Gene	Accession no.
PxTH	*P. xuthus*	Tyrosine hydroxylase	AB178006
BmTH	*B. mori*	Tyrosine hydroxylase	AB439286
DmTH	*D. melanogaster*	Tyrosine hydroxylase	AAF50648
MmTH	*Mus musculus*	Tyrosine hydroxylase	NP033403
HsTH	*Homo sapiens*	Tyrosine hydroxylase	NP000351
DmPAH	*D. melanogaster*	Phenylalanine hydroxylase	CAA66798
PxGTPCHI	*P. xuthus*	GTP cyclohydrolase I isoform B	AB220982
BmGTPCHI	*B. mori*	GTP cyclohydrolase I isoform B	AB439288
DmGTPCHI	*D. melanogaster*	GTP cyclohydrolase I	AY382624
HsGTPCHI	*H. sapiens*	GTP cyclohydrolase I	AAB23164
MmGTPCHI	*M. musculus*	GTP cyclohydrolase I	NP_032128
DmEbony	*D. melanogaster*	Ebony	AAF55870
AgEbony	*Anopheles gambiae*	Ebony	EAA03788
AmEbony	*Apis mellifera*	Ebony	XP392634
PxEbony	*P. xuthus*	Ebony	AB195255
BmEbony	*B. mori*	Ebony	AB439000
NRPS-COG1020	*Crocosphaera watsonii*	Nonribosomal peptide synthetase modules and related proteins	ZP00177120
NRPS-Pseudomonas	*Pseudomonas syringae*	Nonribosomal peptide synthetase, terminal component	AAO58141
DmYellow-f2	*D. melanogaster*	Yellow-f2	NP_650247
DmYellow-f	*D. melanogaster*	Yellow-f	NP_524335
AedesDCE	*Aedes aegypti*	Dopachrome conversion enzyme	AAL85599
BmYellow-fa	*B. mori*	Yellow-fa	DQ358080
BmYellow-fb	*B. mori*	Yellow-fb	DQ358082
BmYellow-f2	*B. mori*	Yellow-f2	DQ358084
PxYellow	*P. xuthus*	Yellow	AB195254
BmYellow	*B. mori*	Yellow	AB438999
DmYellow	*D. melanogaster*	Yellow	AAF45497
AgYellow	*A. gambiae*	Yellow	EAA12085
DmYellow-b	*D. melanogaster*	Yellow-b	NP_523586
DmYellow-c	*D. melanogaster*	Yellow-c	AAQ09899
BmYellow-c	*B. mori*	Yellow-c	DQ358081
DmYellow-h	*D. melanogaster*	Yellow-h	NP_651912
DmYellow-g2	*D. melanogaster*	Yellow-g2	NP_647710
DmYellow-g	*D. melanogaster*	Yellow-g	NP_523888
DmYellow-e	*D. melanogaster*	Yellow-e	NP_524344
BmYellow-b	*B. mori*	Yellow-b	DQ358083
DmYellow-k	*D. melanogaster*	Yellow-k	NP_648772
DmYellow-e3	*D. melanogaster*	Yellow-e3	NP_650288
DmYellow-e2	*D. melanogaster*	Yellow-e2	NP_650289
DmYellow-d2	*D. melanogaster*	Yellow-d2	NP_611788
DmYellow-d	*D. melanogaster*	Yellow-d	NP_523820
BmYellow-d	*B. mori*	Yellow-d	DQ358079

brown markings in the latter part of the molting period (Futahashi and Fujiwara 2005). To determine the correlation between Bombyx larval coloration and *yellow* and/or *ebony* genes, we investigated the temporal expression patterns of *yellow* and *ebony* mRNA in the epidermis by Northern hybridization. On the basis of the visible characteristics, 10 morphological larval stages [A, B, C1, C2, D1, D2, D3, E1, E2 (A–E, fourth instar larval stage), and F (fifth instar larval stage)] could be distinguished and were referred to as the "spiracle index." D1 is a stage when head capsule slippage occurs, and ecdysteroid titer declines during E1 and E2 stages (Kiguchi and Agui 1981). In the wild-type strain, faint *yellow* expression was detected at E1 and weak *ebony* expression was detected at E2 (Figure 4A, left). To analyze *yellow* expression more clearly, we performed Northern hybridization using the p^s (striped) strain, which has black stripes on each segment (Figure 4A; Fujiwara *et al.* 1991). In the p^s strain, *yellow* mRNA was strongly expressed at E1, and *ebony* mRNA was strongly expressed at E2 (Figure 4A, right). Whole-mount *in situ* hybridization showed that *yellow* expression correlated

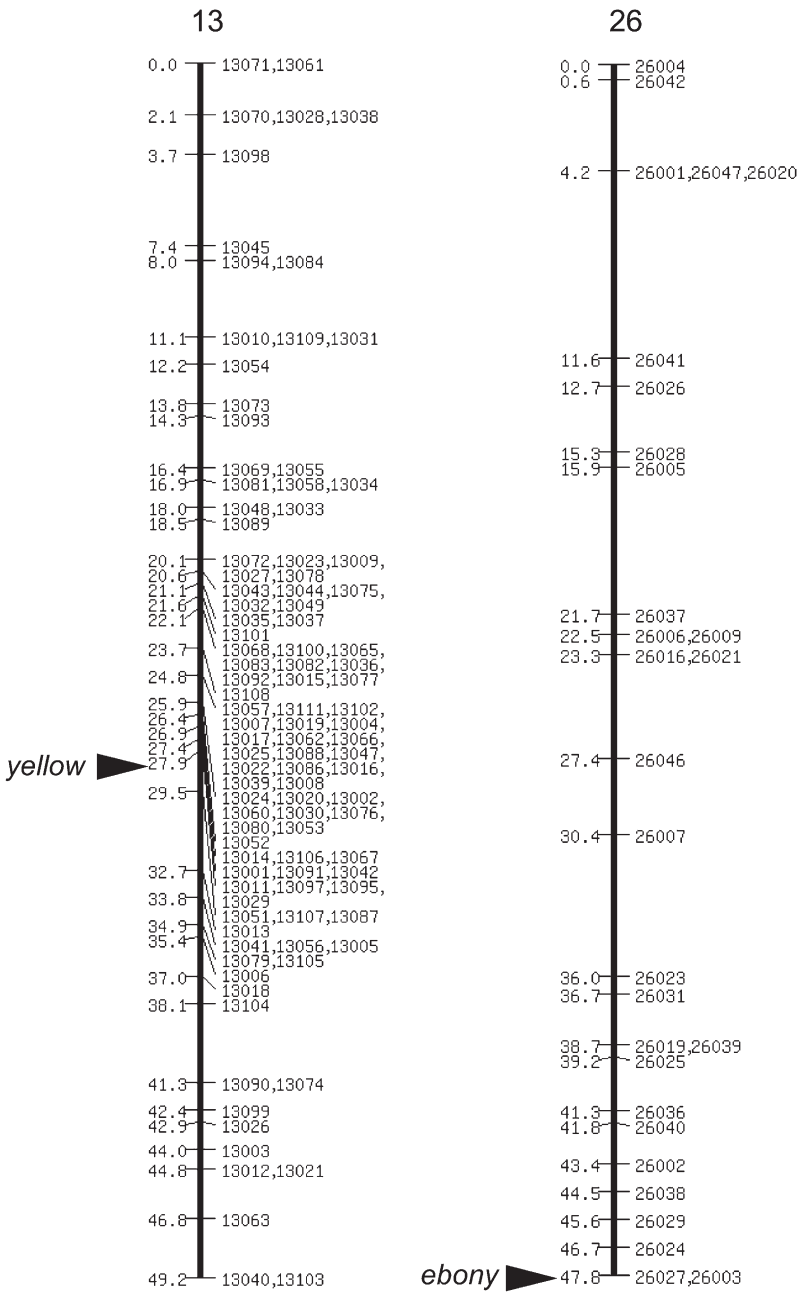

FIGURE 3.—Linkage map between *yellow* and *ebony* and linkage groups 13 and 26, respectively. On the chromosome maps, loci are labeled by position in centimorgans (left) and locus name (right). Linkage maps are the same as in YAMAMOTO *et al.* (2008). Segregation patterns of single-nucleotide polymorphism were surveyed using 190 BC_1 individuals from a single pair mating between a p50T female and an F_1 male (p50T female × C108T male) as previously reported (YAMAMOTO *et al.* 2006). Chromosomal positions of *yellow* and *ebony* genes are indicated by arrowheads.

strongly with the presumptive black markings (Figure 4B) and was not detected in the white striped region (arrowheads in Figure 4B), similar to *P. xuthus* larvae (FUTAHASHI and FUJIWARA 2007), whereas *ebony* expression was broadly detected (data not shown). We next analyzed the tissue specificity of *yellow* and *ebony* mRNA. In addition to the epidermis, *yellow* transcripts were detected in fat body and tracheae during the molting period, but not during the intermolt period (Figure 4C). By RT–PCR analysis, *ebony* transcripts were also detected in fat body and Malpighian tubules even during the intermolt period (Figure 4D). We also investigated numerous Bombyx EST libraries (MITA *et al.* 2003; OKAMOTO *et al.* 2008) and found that *yellow* transcripts were found in compound eyes, epidermis, and wing disc libraries, while *ebony* transcripts were not found in any library (data not shown).

Mutations of the *yellow* gene in the *ch* mutant: We determined the complete cDNA sequences of the *yellow* genes amplified by RT–PCR and RACE from the wild-type p50T strain and two alleles of *ch* mutants, ch^{k12} and ch^{l11}. In the ch^{l11} strain, we found a 14-bp deletion within exon 4. This deletion causes a frameshift within exon 4 and a premature TAA stop codon (Figure 5A, AB455226). We also confirmed this deletion using genomic DNA in the ch^{l11} strain (AB455232). By RT–PCR, multiple bands were detected in the ch^{k12} strain (supplemental Figure 2). We further identified a cDNA sequence after subcloning and

TABLE 2

Chromosomal mapping of five genes involved in melanin-synthesis pathway

Gene	Chromosome	Position (cM)
TH	1	19.0
DDC	4	29.3
yellow	13	27.9
ebony	26	49.2

Positions in each chromosome are as referred to in Yamamoto *et al.* (2008).

TABLE 3

Linkage analysis of the *yellow* gene

	SNP of *yellow* gene		
Phenotype (genotype)	G/G	A/G	A/A
Black (+/+, *ch*/+)	27	59	0
Red (*ch*/*ch*)	0	0	93

Single nucleotide polymorphism analysis was performed on 179 individuals obtained from an F_2 intercross (heterozygous F_1 females mated to F_1 heterozygous males). The first base of the third intron of *yellow* of wild type (p50T strain) was guanine while that of ch^{k12} was adenine.

found that four cDNAs were produced in the ch^{k12} strain (Figure 5A, AB455227–AB455230). All of these cDNAs had aberrant exon 3's (Figure 5A). Type 1 cDNA was spliced from exon 2 to exon 4 and did not have exon 3 (the stop codon position is identical to that of the wild type). Type 2 cDNA had a 167-bp-longer exon 3 than the wild type, which caused a premature TGA stop codon in exon 3. Type 3 cDNA was spliced before normal intron 3 and had a 29-bp-shorter exon 3 than the wild type, which caused a premature TGA stop codon in exon 4. Type 4 cDNA had an 88-bp-longer exon 3 than the wild type, which also caused a premature TGA stop codon in exon 4. To clarify the cause of an abnormal exon 3, we also investigated the genomic sequence of the ch^{k12} strain (AB455233) and found that the first base of intron 3 is substituted from G to A in ch^{k12} (Figure 5A, bottom, denoted by asterisk), which violated the GT/AG rule. This disturbance possibly causes inaccurate splicing (new splice junctions were canonical splice junctions as shown in Figure 5A), which results in multiple cDNA production in ch^{k12}. These results demonstrate that the two alleles ch^{k12} and ch^{l11} encode nonsense-mutated *yellow* genes (Figure 5A).

To provide information on the function of Yellow proteins, we compared Yellow amino acid sequences in *B. mori*, *P. xuthus*, and *D. melanogaster*. Amino acid sequences of Yellow proteins were highly conserved among these three species in the major royal jelly protein conserved motif (Yellow box in supplemental Figure 3) and the N-terminal region except for the signal peptide sequence (dotted line in supplemental Figure 3). All mutant proteins disrupted a large part of a major royal jelly protein conserved motif (supplemental Figure 3). Yellow had two potential *N*-glycosylation sites (Geyer *et al.* 1986; Drapeau 2003) in all three species, and mutated Yellow proteins (except for type 1 of ch^{k12} mutant) lacked both of these sites (types 2, 3, and 4 of the ch^{k12} mutant) or one of these sites (ch^{l11} mutant). These results suggest that a major royal jelly protein conserved motif including two potential *N*-glycosylation sites is critical for Yellow function.

Mutations of the *ebony* gene in the *so* mutant: We determined the complete cDNA sequences of the *ebony* genes amplified by RT–PCR and RACE from the wild-type p50T and so^{i41} strains. In so^{i41} strains, we found large deletions in the C-terminal regions of the *ebony* ORF (Figure 5B, AB455231). By RT–PCR, we confirmed the same deletion of *ebony* in the other alleles of so^{i10} mutants (supplemental Figure 4) and that the *ebony* sequence of so^{i10} strain is identical to that of so^{i41}, suggesting that these two strains were derived from the same origin. The deletion of 4.7 kb from the middle part of exon 14 to the 5′ region of intron 16 and that of 1 kb in the middle portion of intron 16 produced an aberrant chimeric structure of exon 14 and an abnormal poly(A) site in the so^{i41} mutant (Figure 5B). This aberration caused a fatal change of the C-terminal sequence of the Ebony protein (supplemental Figure 5). We also confirmed this deletion using genomic DNA in so^{i41} (data not shown). These results showed that the functional Ebony protein was not expressed from the *so* alleles.

To provide information on the function of Ebony proteins, we compared Ebony amino acid sequences in *B. mori*, *P. xuthus*, and *D. melanogaster*. Ebony protein has a sequence similarity with nonribosomal peptide synthetase, and several consensus core sequences (A1–A10 and T) have been reported (Richardt *et al.* 2003). Amino acid sequences of these conserved motifs were highly conserved among these three species except for A1 core sequence (green bracket in supplemental Figure 5). We found that amino acid sequences of the C-terminal region (putative amine-selecting domain, green arrow in supplemental Figure 5; Richardt *et al.* 2003) were also conserved among three insect species and that mutated Ebony proteins lacked a large part of this domain (red arrow in supplemental Figure 5),

TABLE 4

Linkage analysis of the *ebony* gene

	PCR fragment of *ebony* gene	
Phenotype (genotype)	1.3 kb and 0.8 kb	0.8 kb only
Brown (*so*/+)	94	0
Black (*so*/*so*)	0	97

PCR analysis was performed on 191 individuals obtained from a backcross (so^{i41} mutant female mated to an F_1 heterozygous male).

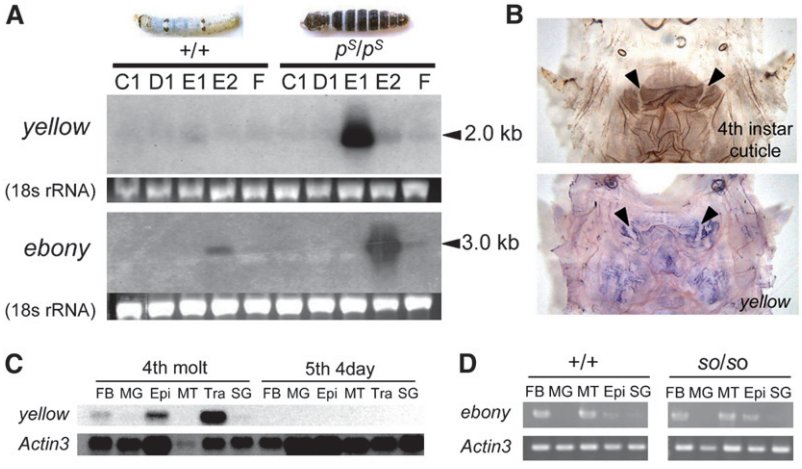

FIGURE 4.—(A) Northern analyses of *yellow* and *ebony* in epidermis during the fourth molting period in wild-type ($+^p$) and striped (p^S) strain larvae. Dorsal view of each strain of larvae is shown above. Ethidium bromide staining of rRNA is shown as loading control. The molting stage (C1, D1, E1, E2, and F) was determined on the basis of the spiracle index (KIGUCHI and AGUI 1981). Arrowheads indicate the positive signal. Expression of both *yellow* and *ebony* are stronger in the p^S strain than in the $+^p$ strain. (B) Spatial expression of *yellow* mRNA in the p^S strain at the thoracic segments. The cuticular pigmentation pattern of the fourth instar larva is shown above. *yellow* expression coincided with the black regions and was not detected at the white striped region (arrowheads). (C) Northern analysis of *yellow* mRNA from several tissues of wild type (p50T strain) at fourth molt or mid-fifth larvae. FB, fat body; MG, midgut; Epi, epidermis; MT, Malpighian tubules; Tra, tracheae; SG, silk gland. *Actin3* is shown as a control. (D) RT–PCR analysis of the *ebony* mRNA in tissues in the V3 (day 3, fifth instar) larvae of wild type (p50T strain) and homozygous *so* strain (so^{i41}/so^{i41}). FB, fat body; MG, midgut; MT, Malpighian tubules; Epi, epidermis; SG, silk gland. *Actin 3* is shown as control.

suggesting that the C-terminal region is critical for Ebony function.

DISCUSSION

Several lines of evidence strongly suggest that *yellow* and *ebony* are responsible for the Bombyx color pattern mutants, *ch* and *so*, respectively. First, *yellow* and *ebony* perfectly cosegregated with the *ch* and *so* loci, respectively (Figure 3). Second, severe defects within the *yellow* ORF existed in the *ch* mutant, and large deletions within the *ebony* ORF existed in the *so* mutant. In both cases, nonsense-mutated proteins were produced (Figure 5; supplemental Figures 3 and 5). Third, expression timing of both genes was associated with the molting

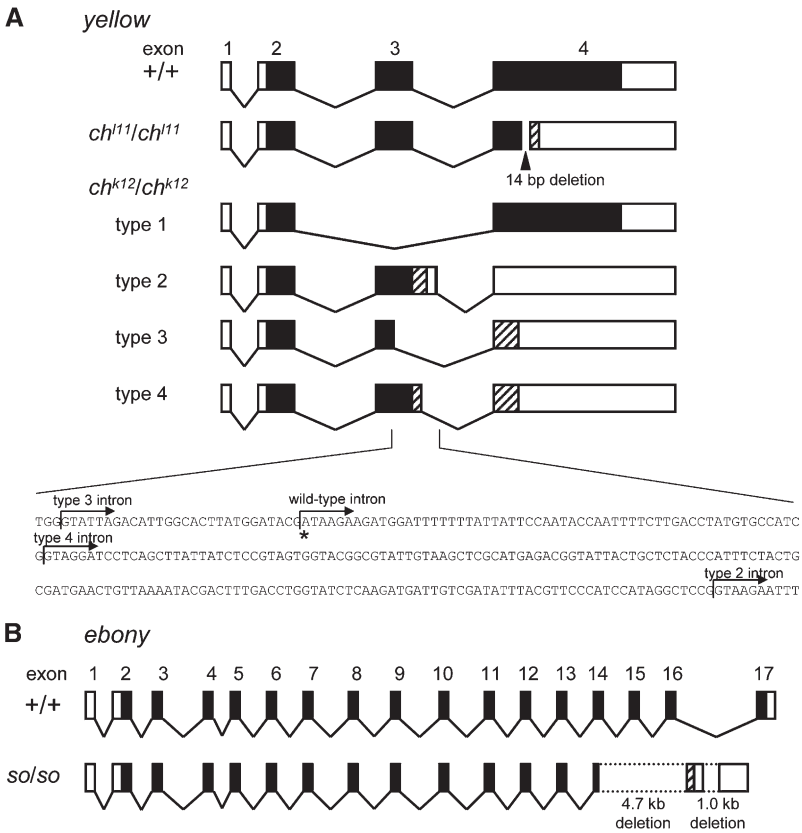

FIGURE 5.—(A) *yellow* cDNA comparison among wild-type and *ch* mutants. *yellow* cDNA of ch^{l11} has a 14-bp deletion in the fourth exon. In ch^{k12}, the splicing site of intron 3 is mutated (below), and four types cDNA were cloned for *yellow*. Asterisk indicates the mutation site at the first base of intron 3. Arrows indicate splicing sites of intron 3 in ch^{k12} strain (types 2, 3, 4). Solid boxes, open reading frames; open boxes, untranslated regions; striped boxes, aberrant open reading frames; diagonal lines, introns. (B) *ebony* cDNA comparison between wild type and *so* mutant. The large deletion causes a fatal change of the C-terminal amino acid sequence of the Ebony homolog protein. Solid boxes, open reading frames; open boxes untranslated regions; striped box, aberrant open reading frame; diagonal lines, introns; dotted lines, deleted region.

period when cuticular pigmentation occurs, and distribution of *yellow* mRNA coincided with the black markings (Figure 4). To our knowledge, this is the first report of melanin-synthesis genes being responsible for color pattern mutants in Lepidoptera. Our results suggest a conserved role of Yellow and Ebony proteins and the potential involvement of both genes in the black color pattern evolution in Lepidoptera.

Yellow function of black color pattern in insects: In *D. melanogaster*, Yellow protein is necessary for normal pigmentation and the distribution of Yellow prefigures adult pigmentation patterns in accordance with species-specific black color pattern (Wittkopp *et al.* 2002a,b; Gompel *et al.* 2005). Mutations in the *yellow* gene produce an altered form of melanin, which results in the light "Yellow" coloration instead of the normal black coloration (Geyer *et al.* 1986; Wright 1987; Wittkopp *et al.* 2002a). Yellow protein may act in the melanin-synthesis pathway downstream from dopa and/or dopamine (Gibert *et al.* 2007), although the precise function of Yellow remains unclear (Drapeau 2003; Gibert *et al.* 2007).

Other than Drosophila, Yellow protein is thought to be involved in larval pigmentation patterns in *P. xuthus* because *yellow* expression coincides with the stage-specific black markings (Futahashi and Fujiwara 2007). Together with our results, *yellow* determines the black pigmentation pattern among insects other than Drosophila and may be associated with black color pattern evolution. In *P. xuthus* larvae, *yellow* expression is restricted to the middle stage of the larval molting period, and topical application of ecdysteroid promotes *yellow* expression (Futahashi and Fujiwara 2007). In *B. mori*, *yellow* expression was also restricted to the middle stage of the larval molting period (Figure 4, A and C), suggesting that regulation of ecdysteroid on *yellow* expression is conserved among lepidopteran species. Because black pigmentation is often regulated by ecdysteroid titer in Lepidoptera, *yellow* is a candidate for connecting ecdysteroid signaling with black pigmentation, like dopa decarboxylase (Hiruma *et al.* 1995; Futahashi and Fujiwara 2007).

Unlike *D. melanogaster*, *yellow* transcripts were found in various tissues, including the compound eye. The coloration of adult compound eye was normal in mutant strains, suggesting that *yellow* is not involved in eye coloration in the silkworm. Because the cDNA library for compound eyes was constructed using mixed stages from fifth instar to pupal (Mita *et al.* 2003), one possibility is that *yellow* is associated with pupal cuticular coloration at compound eyes.

Yellow proteins and Major royal jelly proteins (apalbumin) form a large protein family (Albert and Klaudiny 2004). In *D. melanogaster*, 14 Yellow protein genes have been reported (Drapeau 2001). The *yellow-f* and *yellow-f2* genes have dopachrome-conversion enzyme activity that likely plays an important role during melanin biosynthesis in *D. melanogaster* (Han *et al.* 2002). In *B. mori*, seven Yellow protein genes have been reported (Xia *et al.* 2006). Other *yellow* genes of *B. mori* may also function in pigment synthesis as Drosophila *yellow-f* does and may be responsible for other color pattern mutants.

Ebony function of color pattern in insects: Ebony combines β-alanine with dopamine to form *N*-β-alanyldopamine (NBAD), which is the precursor for a light-colored pigment found in the cuticles of many insect species (Fukushi and Seki 1965; Wright 1987). In the adult abdominal pigmentation of *D. melanogaster*, Ebony expression is detected uniformly and masks melanization (Wittkopp *et al.* 2002a). The difference in expression level of *ebony* is responsible for the variation in thoracic trident pigmentation between the two representative lines in *D. melanogaster* (Takahashi *et al.* 2007), and DNA polymorphism of *ebony* has a clear association with the level of abdominal pigmentation (Pool and Aquadro 2007). Differences in the expression levels of Ebony protein also correlate with the intensity of adult pigmentation between *Drosophila americana* and *Drosophila novamexicana* (Wittkopp *et al.* 2003). In the *ebony* mutant of *D. melanogaster*, dopamine cannot be converted to NBAD, but instead follows an alternate pathway, producing a black pigment. The adult body color of the *ebony* mutant is therefore very dark (Fukushi 1967; Wright 1987; Wittkopp *et al.* 2002a).

Other insect species also require β-alanine for cuticle tanning. Notably, Fukushi and Seki (1965) demonstrated that β-alanine was found in the pupal sheaths of wild strains of Bombyx, but not in a *so* mutant of *B. mori*, as in an *ebony* mutant of *D. melanogaster* (Fukushi 1967). The similarity of pupal amino acid composition also supports our results; we proposed that Bombyx *so* is the ortholog of Drosophila *ebony* (Figure 2). Black mutants of the housefly, *Musca domestica*, also lack β-alanine in their pupal sheaths (Fukushi and Seki 1965). The cuticle of the red flour beetle, *Tribolium castaneum*, is normally a rust-red color as a result of low levels of dopamine combined with high levels of NBAD. A black mutant strain has much higher levels of dopamine and greatly reduced amounts of β-alanine and NBAD (Kramer *et al.* 1984). Ebony may be responsible for black mutants among insects.

In Lepidoptera, the Ebony protein is involved in the production of yellow papiliochrome in *Papilio glaucus* adult wing (Koch *et al.* 2000) and reddish-brown pigments in *P. xuthus* larval body markings (Futahashi and Fujiwara 2005). The activity of Ebony is concerned with light pigmentation in both cases. In *B. mori* larvae, however, larval cuticle is mainly transparent, and Ebony expression was detected uniformly, suggesting that Ebony masks melanization as in Drosophila (Wittkopp *et al.* 2002a).

Other color pattern mutants in Bombyx: There are many spontaneous color pattern mutants (mainly in larvae) in *B. mori* (Banno *et al.* 2005). Bombyx color pattern mutants are good examples of how similar

phenotypes are caused by different loci. Similar to *so* mutants, another mutant, *black pupa* (*bp*; 40.3 cM of linkage group 11), produces black pupa (Hashiguchi *et al.* 1965), although β-alanine is found in the pupal sheaths (Fukushi and Seki 1965). Other than the *ch* mutant, reddish-brown pigmentation of neonate larvae is also characteristic of four other mutant strains: *sex-linked chocolate* (*sch*), *Dominant chocolate* (*Ia*), *chocolate-2* (*ch-2*), and *maternal chocolate* (*cm*). All of these loci have been mapped on the Bombyx linkage group: *sch*—21.5 cM of linkage group 1; *Ia*—22.1 cM of linkage group 9; *ch-2*—0.0 cM of linkage group 18; and *cm*—41.9 cM of linkage group 20 (Banno *et al.* 2005). We found that the TH gene, the first enzyme of the melanin-synthesis pathway, was located to 19.0 cM of linkage group 1 (Table 2), which is near the *sch* locus. Further studies are needed to determine whether TH is associated with the *sch* mutant.

Notably, adult color patterns are affected only slightly in both *ch* and *so* mutants. In many cases, adult and larval color patterns are regulated independently (Banno *et al.* 2005). In two mutants, *Black moth* (*Bm*; 0.0 cM of linkage group 17) and *melanism* (*mln*; 41.5 cM of linkage group 18), the coloration of adult wings is overall black, but the *Bm* larval coloration is normal. The responsible genes of these two mutants may be associated with adult wing color variation. By mating *ch* and/or *so* mutants with other mutant strains, we can see how melanin-synthesis enzymes play a role in color pattern formation and how they interact with other color pattern genes. Recently, positional cloning of several mutants has been completed in *B. mori* (Ito *et al.* 2008; Sato *et al.* 2008). Exhaustive identification of these color pattern mutants will shed light on the mechanisms of color pattern evolution in Lepidoptera.

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