Title running head: *Yellow* function in black soldier fly

Genome-wide identification of *yellow* gene family in *Hermetia illucens* and functional analysis of *yellow-y* by CRISPR/Cas9

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Abstract

The *yellow* gene family plays a crucial role in insect pigmentation. It could potentially be used as a visible marker gene in genetic manipulation and transgenic engineering in several model and non-model insects. Sadly, *yellow* genes have rarely been identified in Stratiomyidae species and the functions of *yellow* genes are relatively unknown. In the present study, we first manually annotated and curated 10 *yellow* genes in the black soldier fly (BSF), *Hermetia illucens* (Stratiomyidae). Then, the conserved amino acids in the major royal jelly proteins (MRJP) domain, structural architecture, and phylogenetic relationship of *yellow* genes in BSF were analyzed. We found that the BSF *yellow* genes *yellow-y*, *-c*, and *-f* are expressed at all developmental stages, especially in the prepupal stage. Using the CRISPR/Cas9 system, we successfully disrupted *yellow-y*, *-c* and *-f* in BSF. Consequently, a mutation in *yellow-y* clearly resulted in a pale-yellow body colour in the prepupae, pupae and adults instead of the typical wild type black body colour. However, a mutation in *yellow-c*/*-f* genes did not result in any insects colour change when compared with the wild type. Our study indicates that BSF *yellow-y* gene plays a role in body pigmentation, providing an optimal marker gene in genetic manipulation of BSF.

Keywords *Hermetia illucens*, yellow family, *yellow-y*, pigmentation, CRISPR-Cas9

Introduction

Insect color has crucial biological implications (Popadić & Tsitlakidou, 2021). Compound eye and body color changes are visible indicators of an efficient genetic marker in model organisms (such as *Drosophila melanogaster* and *Bombyx mori*) in genetic research (Wang *et al*., 2013; Gantz & Bier, 2015; Li *et al*., 2021). Colour change is also reflected in the dimorphism of male and female insects and their seasonal adaptation (Rodriguez-Caro *et al*., 2021; Wang *et al*., 2022; Mank, 2023). Furthermore, coloration is closely related to the survival of insects, including cuticle formation, warning coloration, mimicry, aposematism, mating signals, and others (Wittkopp *et al*., 2002; Tsuchida *et al*., 2010; Matsuoka & Monteiro, 2018; Massey *et al*., 2019; Liu *et al*., 2020). Insect coloration is mostly produced by the body wall and its derivatives, but also comes from different subcutaneous tissues or blood (Lu *et al*., 2023). From the origin of color, it can be divided into pigment color, structural color and mixed color. One of the most common pigments found in an insect cuticle is melanin, and some melanin pathway products also take part in cuticle hardening or sclerotization and they play an important role in the darkening and hardening of insect cuticles (Hopkins and Kramer, 1992; Arakane *et al*., 2010; Andersen, 2010; Connahs *et al*., 2022).

Insect Yellow proteins are unique to insects. They contain approximately 300 amino acid major royal jelly proteins (MRJP) domain (Drapeau *et al*., 2006). The *yellow* gene family is a large and diverse group and represents a rapidly evolving gene family having pleiotropic functions (Ferguson *et al*., 2011). In *D. melanogaster*, a *yellow* gene mutation does not only affects its morphological features, such as melanin pigmentation and cuticle formation, but also affects its behavior, such as decrement in locomotor activity or sexual behavior in males (Walter *et al*., 1991; Wittkopp, 2002; Chen *et al*., 2018; Massey *et al*., 2019). In addition, *yellow-f* has a dopachrome conversion enzyme activity (Han *et al*, 2002). In *Bicyclus anynana*, *yellow* gene is required for butterfly scale color and morphology and also male sexual behaviors (Zhang *et al*., 2017; Matsuoka, 2018). In *Tribolium castaneum*, RNAi mediated transcript knockdown demonstrated that *yellow-y* is essential for melanin production in the hindwings, *yellow-*

f is required for adult cuticle sclerotization, and *yellow-e* gene is involved in body coloration and anti-dehydration (Arakane *et al*., 2010; Noh *et al*., 2015). In *B. mori* and several Lepidopteran pests, the *yellow-y* has been identified to be involved in synthesizing black melanin (Chen *et al*., 2018; Liu *et al*., 2020; Han *et al*., 2021; Shirai *et al*., 2021; Wang *et al*., 2021). Taken together, although the *yellow* family genes appears to have an important and diverse functions, the physiological functions of Yellow proteins are largely unknown. Therefore, whether the function of black soldier fly (BSF), *Hermetia illucens yellow* family genes and the phenotype of *yellow* knock-out were different from previous studies or not is worth exploring.

The BSF is important for nutrient-recycling through bioconversion of organic waste into biomass, and promoting the formation of circular economy (Mazza *et al*., 2020; Zhan *et al*., 2020). Most importantly, the BSF larvae accumulates large amounts of protein and fat in the process of converting organic waste (Somroo *et al*., 2019). Hence, the prepupae and pupae are rich in various nutrients required for animal growth and development, such as amino acids, calcium and chitosan trace elements. Thus BSF could serve as a high-quality protein feed source for livestock and aquaculture to solve the shortage of protein resources (Raksasat *et al*., 2020; Liu *et al*., 2022; Lopes *et al*., 2022). Although high-yielding BSF strains can be achieved through CRISPR-based genome editing techniques and transgenic engineering (Zhan *et al*., 2020; Kou *et al*., 2022), optimal genetic marker genes are still lacking in the process of genetic manipulation. Therefore, in the current study, we first identified the *yellow* family genes in BSF. Then the evolutionary relationship, gene structure, and conserved domains of *yellow* members were analyzed in BSF. In addition, the expression profiles of BSF *yellow* family genes are investigated at different developmental stages and in adult tissues. We then used the CRISPR/Cas9 genome-editing system to perform functional analysis of BSF *yellow-y*, *yellow-c* and *yellow-f*. Our results demonstrates that *yellow-y* gene plays a role in body pigmentation and provides a potential marker gene in the breeding process.

Materials and Methods

Insect rearing

The black soldier fly strain was originally sampled in Wuhan, China (30.6°N, 114.4°E) (Zhan *et al*., 2020). Larvae were reared in an incubator at 25 ± 1°C, 40 ± 5% relative humidity and a 16: 8 h light/dark photoperiod, and fed with a mixture of wheat and water artificial diet. Adult flies were allowed to mate in a square cage (30 × 30 × 30 cm^3) and fed with a 5% honey solution.

Manual annotation and cloning of yellow family genes in BSF

Yellow family genes of *D. melanogaster* were curated with reference to both a previous study (Drapeau, 2001) and the annotation available on FlyBase (http://flybase.org/). The protein sequence of each gene was subjected to BLASTP against BSF gene sets (Zhan *et al*., 2020; Generalovic *et al*., 2021). Identified genes with significant hit (E value < $1e^{-5}$) were reversely BLASTP against the *D. melanogaster* gene set to check the reciprocal hit. Meanwhile, TBLASTN search was performed to check the possibility of being missed upon the absence in gene sets.

Using cDNA synthesized from total RNA of an adult fly template, the open reading frame of each putative *yellow* gene was amplified with LA Taq (TaKaRa, China) under the following PCR conditions: 95°C for 5 min, 35 cycles of 94°C for 30 s, 56°C for 30s, 72°C for 70 s, followed by final extension at 72°C for 70 s. The amplified products were subcloned into PMD-18T vector (TaKaRa, China) for sequencing. All primers used in this study are listed in Table S1. The exon and intron boundaries of the *yellow* family genes were acquired from the BSF genomic database (PRJNA547968). The conserved domains in the Yellow family proteins were predicted using InterPro (http://www.ebi.ac.uk/interpro/search/sequence-search).

Phylogenetic analysis of yellow family genes

The amino acid sequences encoded by the *yellow* genes from *D. melanogaster*, *B. mori*, *T. castaneum*, *Aedes aegypti* and *Apis mellifera* have been previously reported (Drapeau,

2001; Xia *et al*., 2006; Arakane *et al*., 2010). Amino acid sequences of Yellow proteins from these five species and the predicted amino acid sequence of BSF were aligned by Clustal W (Thompson *et al*., 1994); the alignment presentation was created with the ClustalX2 software (Larkin *et al*., 2007). The phylogenetic tree was inferred using the maximum likelihood method in MEGA-X with a bootstrap of 1000 replications.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from different development stages and tissues of six individual BSF using RNAiso Plus (Yeasen, China) following the manufacturer's protocol. Then reverse transcription was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, China). qRT-PCR was performed using Hieff® qPCR SYBR Green Master Mix (Yeason, China) according to the manufacturer's protocol on Eppendorf Real-time PCR System Mastercycler® RealPlex (Eppendorf, Germany). A relative quantitative method ($^{\triangle\triangle}$Ct) was used to evaluate the quantitative variation. *RP49* was chosen as the reference gene for qRT-PCR analysis. Primer information is shown in Table S1.

CRISPR/Cas9-based gene editing

We used CRISPR/Cas9 to knockout the target gene in *vivo*. In brief, 23-bp single guide RNAs (sgRNAs) target site were designed based on the consensus target $GGN_{19}GG$. The uniqueness of each target loci in BSF genome was verified using BLASTN. The different sgRNAs were synthesized *in vitro* with MEGAscript T7 kit (Ambion, USA) following the manufacturer's protocol. Then all sgRNAs were purified with phenol: chloroform: isoamylol (25: 24: 1) and stored at -80°C. In addition, the Cas9 protein (TrueCut™ Cas9 Protein v2) was purchased from Thermo Fisher, China.

Female BSF were allowed to lay eggs on corrugated article. Then fresh embryos were arranged in a line on a glass microscope slide as previously described (Kou *et al*., 2022). A mixture of sgRNAs (300 ng/μL) and Cas9 protein (330 ng/μL) were injected into the posterior pole of each embryo with glass needles using a microinjection manipulation system (IM300 Narishige, Japan). All operations were completed within 2 h after

oviposition. The injected embryos were incubated in a chamber at 25 °C and 65 ± 5% relative humidity until hatching.

Mutation detection

Briefly, to confirm mutagenesis of the *yellow-y*, *yellow-c* and *yellow-f* locus, the genomic DNA was extracted with standard sodium dodecyl sulfate lysis-phenol buffer from injected eggs, larvae, pupae or adults carrying phenotypes. PCR was performed to amplify the fragments surrounding the different target-sites from the genomic DNA samples. The amplified products were respectively ligated into the PMD-18T vector (TaKaRa, China) and sequenced. The specific primer sequences are listed in Table S1

Phenotype screening

Hatched larvae were collected and fed with fresh artificial diet. The mutant phenotype of larvae, pupae, and adults were checked and imaged digitally under a digital camera (Nikon DS90, Japan). Wings and antennae of mutant or wild-type insects were dissected from the adults and subjected to morphological investigation under a stereomicroscope (Nikon SW-2B/22, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software with an independent Student's *t*-test. The data are presented as means ± SEM, and statistical significance was assumed for $p < 0.05$.

Results

Identification and characterization of yellow family genes in BSF

Using our previously assembled BSF genome (Zhan *et al*., 2020), we manually annotated 10 genes encoding proteins homologous to *D. melanogaster* Yellow proteins (Fig. 1A). Sequences of yellow proteins were listed in Table S2. Furthermore, the exon and intron boundaries of each BSF *yellow* gene were acquired from analysis of BSF genomic and transcriptomic database. Although the exon/intron structure of *yellow* family genes in BSF are not completely consistent, all the Yellow proteins contain

conserved MRJP domains (Fig. 1). These results suggest that, the function of *yellow* genes may be similar to that of other insect species.

Phylogenetic relationship of yellow proteins in six insect species

To elucidate the evolutionary relationships of Yellow protein in BSF and other five insects, we reconstructed an ML phylogenetic tree with 70 full-length yellow amino acid sequences from six species (Fig. 2A). In this tree, the yellow proteins in groups *y*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, and *x* are respectively classified into separate clades with significant bootstrap values, suggesting that yellow proteins are well conserved across these insect species. The 10 genes encoding BSF yellow proteins were named according to their close relationships to characterize orthologs of other species (Fig. 2A). Particularly, *A. mellifera* yellow proteins grouped into a single branch. In addition, all sequences of BSF *yellow* genes were validated by end-to-end PCR.

Stage and tissue-expression profiles of yellow family genes in BSF

To gain further insights into putative function of BSF *yellow* genes, their transcription profiles were investigated at different developmental stages and adult tissues by using qPCR. *Hillyellow-y*, *-c*, *-f*, *-b*, *-d*, and *-d2* were constitutively expressed in all stages. The expression level of *yellow-y*, *-c*, and *-f* were significantly increased from 5th instar larvae to prepupae (Fig. 2B). We also found *yellow-e*, *-g*, *-g2* and *-h* were not expressed or expressed only at a low level in larval stages (CT values were more than 30) (Fig. 2B). Besides, *yellow-c* and *yellow-d* showed significant difference between the female and male adults (Fig. 2B). We further evaluated the spatial expression profiles of *yellow* genes in different tissues of mature adults. All 10 *yellow* genes were expressed in epidermis, although *yellow-g2* mRNA was detected at a relatively low expression level (CT values were more than 30) (Fig. 2C). Interestingly, *yellow-y* was highly expressed in all tissues (Fig. 2C).

CRISPR-Cas9 mediated mutagenesis of yellow family genes in BSF

We used the CRISPR/Cas9 system to investigate the function of the *yellow-y*, *yellow-c* and *yellow-f* genes in BSF. All of which are highly expressed throughout the insect's

developmental cycle, especially during the prepupal stage. A single target site in exon was respectively designed for CRISPR/Cas9-targeted mutagenesis (Fig. 3A and Fig. S1-S2). We then co-injected the sgRNAs and Cas9 into 189, 171 and 201 newly laid eggs to disrupt *yellow-y*, *yellow-c* and *yellow-f*, respectively. All eggs hatched at 96 h after injection. Hatching rates were 74%-83% for different injected groups (Table 1), indicating that microinjecting the different sgRNAs had no significant effect on embryonic development. From the 10 eggs or first-instar larvae randomly selected for sequencing after injection, we found different types of indels (Fig. 3B-3C and Fig. S1-S2), indicating that the CRISPR/Cas9 system effectively mutagenized *yellow-y*, *yellow-c* and *yellow-f*.

The mutant of yellow-y gene induced yellow color pigmentation of pupae and adults

In order to clarify the physiological functions of *yellow-y*, *yellow-c* and *yellow-f* during the development of BSF, we compared the phenotypes of animals in wild-type with three mutant groups at all developmental stages. After hatching, the embryonic and neonate larvae were counted to investigate if *yellow* family gene knock-out caused hatching defects. We found that, the embryonic larvae were alive inside the chorions and exhibited normal development relative to the wild-type larvae (data not shown). Next, we observed that the head and body wall of the wild-type BSF exhibited an oyster white color throughout the larval stages (Fig. 4A). The *yellow-y*, *yellow-c* and *yellow-f* mutants had normal pigmentation (Fig. 4A). In the prepupal stages, the expression of *yellow-y*, *yellow-c* and *yellow-f* were significantly increased, and we also observed the color of both head and body wall of wild-type BSF becomes darker throughout the prepupae and pupae stages (Fig. 4B and Fig. 5A). In *yellow-y* mutants, the body surface of prepupae and pupae remained a light-yellow color. However, no obvious difference in color was observed in *yellow-c* and *yellow-f* mutants and wild-type insects in prepupal and pupal stage (Fig. 4B and Fig. 5A). In the adults, wild-type BSF possess the black body and a pair of black wings and antennae (Fig. 5B and Fig. 6). Interestingly, *yellow-y* mutant body, wings and antennae presented pale yellow, indicating a correlation between the expression of *yellow-y* and normal pigmentation. However, the

yellow-c and *yellow-f* mutant presented normal pigmentation of antennae and wings compared with wild-type BSF (Fig. 5B and Fig. 6). Taken together, our results suggested that *yellow-y* is essential for pigmentation in BSF but *yellow-c* and *yellow-f* are not.

Mutation of yellow-y is inheritable

To analyze the transmission of mutations to the subsequent generation, the *yellow-y* female mutants were crossed with *yellow-y* male mutants. In total, 25 pairs of flies were crossed and laid eggs. Ultimately, we obtained several pale-yellow flies (Fig. 7A-C), and the germline transmission frequency of CRISPR/Cas9 induced mutations at *yellow-y* locus was calculated to be 32% (Table 1). Meanwhile, to verify the consistency between the phenotype mutants and the genotype mutants, genomic DNAs from prepupae, pupae and adults were respectively amplified and sequenced. As expected, deletions and insertions occurred at the targeted site in the *yellow-y* locus of these insects (Fig. 7D), confirming the function of *yellow-y* in pigmentation of body wall at pupal and adult stages.

Discussion

The *yellow* gene family exhibits a great functional diversity, with roles in cuticle formation, melanin pigmentation, competitive mating ability, locomotor activity, and butterfly scale color and morphology (Walter *et al*., 1991; Wittkopp, 2002; Chen *et al*., 2018; Hinaux *et al*., 2018; Massey *et al*., 2019; Wang *et al*., 2021). However, there has been no systematic identification or characterization of the evolutionary relationships and physiological functions of *yellow* family genes in BSF. Therefore, we performed a genome-wide systemic analysis of the *yellow* genes in BSF, including sequence phylogeny, gene structure, conserved domain, and expression profiling. We also functionally analyzed the *yellow-y* gene using the CRISPR/Cas9 system to generate loss-of-function mutants. This comprehensive analysis of *yellow* family genes and their evolutionary and functional characteristics has provided insights into the function of *yellow* gene in Stratiomyidae.

Yellow gene repertoires have been identified at genomic level in several insects, including 14 in *D. melanogaster*, 7 in *B. mori*, 14 in *T. castaneum,* 14 in *A. ipsilon*, 11 in *Spodoptera frugiperda* and *S. litura* (Drapeau, 2001; Xia *et al*., 2006; Arakane *et al*., 2010; Chen *et al*., 2018; Liu *et al*., 2020; Han *et al*., 2021). Here, we have identified 10 *yellow* genes in BSF. Although, members of a *yellow* gene repertoire are not identical, they do share a conserved MRJP domain. In addition, the expression patterns of *yellow-y*, *yellow-c* and *yellow-f* was very consistent in all the life stages examined, indicating that they may have a similar physiological function in the development process. Unexpectedly, mutation of *yellow-c* and *yellow-f* had no observable effects on cuticle pigmentation in pupae and adults, yet we realized that, a disruption of *yellow-y* produced pale yellow prepupae, pupae and adults. Unfortunately, we cannot adequately explain the spatiotemporal expression of the *yellow* family genes, thus, further research is necessary to draw a firm conclusion.

The *yellow* genes are thought to be involved in the biosynthesis pathway of melanin in insects (Biessmann, 1985; Wittkopp *et al*., 2002; Wittkopp & Beldade, 2009). In Lepidoptera, *yellow-y* gene serves crucial functions in larval pigmentation and color patterns across broad taxa of Lepidopteran insects (Shirai *et al*., 2021). In addition, *yellow-y* appears to be necessary for egg hatching, segmentation and molting of larvae in *S. litura* (Liu *et al*., 2020), whereas *yellow-y* has a vital role in the development and reproduction of *S. frugiperda* (Han *et al*., 2021). In *A. ipsilon*, *yellow-y* is required for waterproofing of larvae (Chen *et al*., 2018). In Dipteran, nevertheless, *yellow-y* influences mating behavior and pigmentation of *D. melanogaster* (Massey *et al*., 2019). In this study, we have demonstrated that, the *yellow-y* gene is important in BSF prepupae, pupae and adults pigmentation, suggesting that, the *yellow* gene family appear to have pleiotropic functions. In the BSF, these functions are largely unknown and thus need further investigations.

In recent years, TALENs and CRISPR/Cas9 have been exploited to improve SIT in mosquitoes and *Drosophila* (Basu *et al*., 2015; Alphey, 2016; Kaduskar *et al*., 2022). A series of recent reports have revealed that, using the gene drive systems to target sterility

genes causes a complete population suppression in *Culex quinquefasciatus* and *Anophele gambiae* (Feng *et al*, 2021; Taxiarchi *et al*, 2021). This study has proved that, *yellow-y* can potentially be used as a germline transformation marker gene for constructing transgenic BSF. Therefore, our work provides a useful and measurable target in genetically based pest control prototypes. In conclusion, we have identified 10 *yellow* genes and revealed its spatiotemporal expression pattern in BSF. In addition, we have clearly characterized their physiological functions using the CRISPR/Cas9 system in *vivo*. Our results suggest that *yellow-y* is required for cuticle pigmentation in BSF. We anticipate that, this data will provide a novel genetic target for the genetic breeding of economic insect such as BSF.

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Disclosure

All the authors declare no conflicts of interest associated with this work.

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Figure legends

Fig. 1 Characterization of BSF *yellow* family genes. (A) Gene structure of BSF *yellow* family genes. The orange boxes denote exons and black lines representing introns. (B) Domain structures of BSF Yellow proteins with junction amino acid residues numbered. The major royal jelly protein (MRJP) domains are shown in purple boxes.

Fig. 2 Phylogenetic relationship of Yellow proteins in insects and the expression heatmap of *yellow* family genes in BSF. (A) Molecular phylogenetic analyses were conducted using the maximum-likelihood (ML) tree with MEGA-X from Yellow protein amino acid sequences of six species. Insect species are labeled with colors: BSF (red), *D. melanogaster* (black), *T. castaneum* (purple), *A. mellifera* (blue), *A. aegypti* (orange), and *B. mori* (green). Bootstrap values were obtained by 1000 replications. Accession numbers or annotation IDs are provided in the Table S1. (B-C) The expression heatmap of BSF *yellow* genes determined by qPCR in developmental stages (B) and various tissues (C). Data were normalized based on the expression value of each gene in all developmental stages and tissues analyzed. L1, first-instar larvae; L2 second-instar larvae; L3, third-instar larvae; L4, fourth-instar larvae; L5, fifth-instar larvae; PP, prepupae; P, pupae; F, female adults; M, male adults; LG, legs; WG, wings; AN, antennae; EPI, epidermis; FB, fat body; HA, head.

Fig. 3 Targeted mutation of *yellow-y* was induced by CRISPR/Cas9. (A) Schematic

diagram of gene sequences and sgRNA target site in BSF *yellow-y* gene. The target sequence and PAM sequence are showed in green and red, respectively. (B) Representative sequencing chromatograms of PCR products of *yellow-y* from WT and microinjected embryos. The targeted site is highlighted by black lines. (C) Mutant sequences determined by sequencing. The WT sequence was shown at the top. Dashed lines represent the deleted bases. The net change in length is marked at the right of each sequence (−, deletion).

Fig. 4 Phenotypes of the fifth instar larvae and prepupae of the *yellow* G0 mutants. (A) Significant differences in pigmentation were not observed between mutants and wild types at fifth instar larvae. Scale bar: 1 cm. (B)The *yellow-y* mutants show abnormal pigmentation compared to the wild types and other two mutants at prepupal stage. Scale bar: 1 cm.

Fig. 5 Phenotypes of *yellow* G0 mutants in pupal and adult stages. The *yellow-y* mutants show the pale-yellow body pigmentation in pupal and adult stage (A) and (B), while *yellow-c* and *yellow-f* mutants present normal pigmentation consistent with that of the wild types. Scale bar: 1 cm.

Fig. 6 Phenotypes of wings (A) and antennae (B) of wild type and yellow G0 mutant insects. The *yellow-y* mutants show the pale-yellow wings (A) and antennae (B), while *yellow-c* and *yellow-f* mutants present normal pigmentation consistent with that of the wild types. (Wing, scale bar 5 mm; antenna, scale bar 1 mm).

Fig. 7 Phenotypes of *yellow-y* G1 mutants at prepupal (A), pupal (B) and adult (C) stages. The *yellow-y* mutants show abnormal (pale-yellow) pigmentation compared to the wild types. Scale bar 1 cm. (D) Mutant sequences confirmed by sequencing. Dashed lines represent the deleted bases. The net change in length is marked at the right of each sequence (−, deletion).

Table 1. Efficiency of CRISPR/Cas9 mediated mutagenesis

	Cas9/sgRNA concentration (ng/μL)	No. injected embryos	Hatching rate (%)	No. pupae	No. adults	Mutation phenotype rate (%)	Germline mutation rate (%) (N)[1]
yellow-y	200ng/300ng	189	81%	122	101	63%	32% (8)
yellow-c	200ng/300ng	171	74%	98	84	0	0
yellow-f	200ng/300ng	201	83%	130	112	0	0
ddH_2O	-	153	86%	101	86	0	0

[1] The germline mutation rate corresponds to the number of batches from 25 couples that contained G1 mutants.

Supporting Information

Fig. S1 Targeted mutation of *yellow-c* induced by using the CRISPR/Cas9 system. (A) Schematic diagram of sgRNA target site designed in the *yellow-c* locus. (B) PCR analyses of the *yellow-c* mutant insects with mutation checking primers (Table S1). (C) *yellow-c* mutant sequences were confirmed by sequencing.

Fig. S2 Targeted mutation of *yellow-f* induced by using the CRISPR/Cas9 system. (A) Schematic diagram of sgRNA target site designed in the *yellow-f* locus. (B) PCR analyses of the *yellow-f* mutant insects with mutation checking primers (Table S1). (C) *yellow-f* mutant sequences were confirmed by sequencing.

Table S1. Primer sequences used in this study.

Table S2. Amino acid sequences used in the phylogenetic analysis.