Allele-specific knockouts reveal a role for *apontic-like* in the evolutionary loss of larval pigmentation in the domesticated silkworm, *Bombyx mori*

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Running title: Genome editing in *Bombyx mandarina*

Abstract

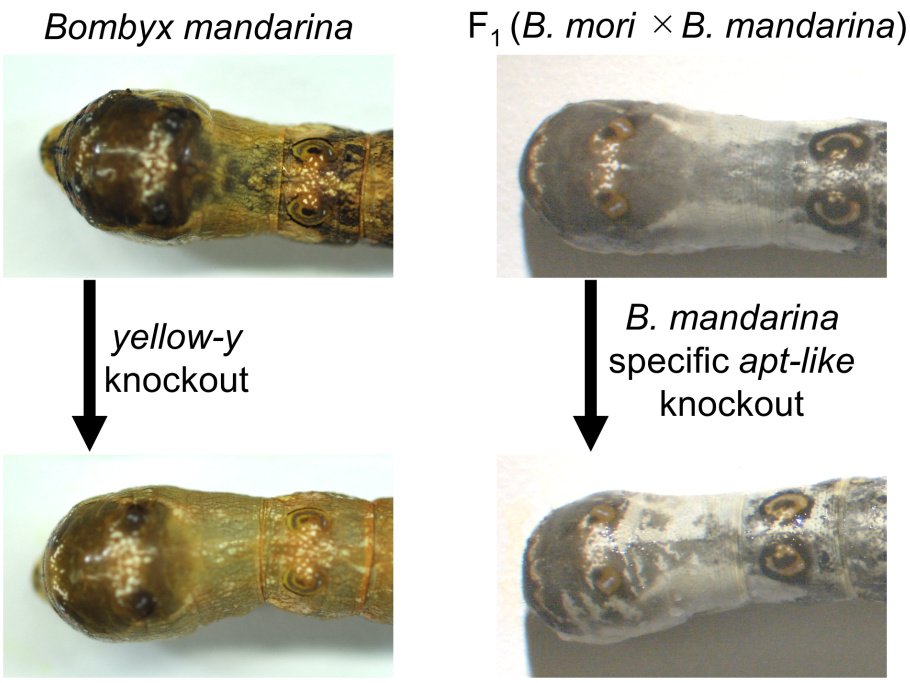
The domesticated silkworm, *Bombyx mori*, and its wild progenitor, *B. mandarina*, are extensively studied as a model case of the evolutionary process of domestication. A conspicuous difference between these species is the dramatic reduction in pigmentation in both larval and adult *B. mori*. Here we evaluate the efficiency of CRISPR/Cas9-targeted knockouts of pigment-related genes as a tool to understand their potential contributions to domestication-associated pigmentation loss in *B. mori*. To demonstrate the efficacy of targeted knockouts in *B. mandarina*, we generated a homozygous CRISPR/Cas9-targeted knockout of *yellow-y.* In *yellow-y* knockout mutants, black body color became lighter throughout the larval, pupal and adult stages, confirming a role for this gene in pigment formation. Further, we performed *allele-specific* CRISPR/Cas9-targeted knockouts of the pigment-related transcription factor, *apontic-like* (*apt-like*) in *B. mori* × *B. mandarina* F_1 hybrid individuals. Knockout of the *B. mandarina* allele of *apt-like* in F_1 embryos results in depigmented patches on the dorsal integument of larvae, whereas corresponding knockouts of the *B. mori* allele consistently exhibit normal F_1 larval pigmentation. These results demonstrate a contribution of *apt-like* to the evolution of reduced pigmentation in *B. mori*. Together, our results demonstrate the feasibility of CRISPR/Cas9-targeted knockouts as a tool for understanding the genetic basis of traits associated with *B. mori* domestication.

Key words: *Bombyx mandarina*, domestication, pigmentation, CRISPR/Cas9, reciprocal hemizygosity test.

Brief abstract

Bombyx mori and its wild progenitor are an important model for the study of phenotypic evolution associated with domestication. As proof-of-principle, we used CRISPR/Cas9 to generate targeted knockouts of two pigmentation-related genes. By generating a homozygous knockout of *yellow-y* in *B. mandarina*, we confirmed this gene's role in pigment formation. Further, by generating *allele-specific* knockouts of *apontic-like* (*apt-like*) in *B. mori* × *B. mandarina* F_1 hybrids, we establish that evolution of *apt-like* contributed to reduced pigmentation during *B. mori* domestication.

Graphical TOC/Abstract



51 **Introduction**

52 The silkworm, *Bombyx mori*, was domesticated over 5000 years ago from its
53 wild progenitor species, *B. mandarina*. Under long-term artificial selection, *B. mori*
54 acquired various characteristics suitable for sericulture. For example, the weight of the
55 cocoon shell of *B. mori* is much higher than that of *B. mandarina* (Ômura 1950, Li *et al.*
56 2017, Fang *et al.* 2020). In addition, *B. mori* moths lost their flying ability due to the
57 degeneration of their flight muscles and a reduction in wing stiffness (Lu *et al.* 2020).
58 Among the most conspicuous domestication-associated traits is a marked reduction in
59 pigmentation in *B. mori* larvae and adults relative to *B. mandarina* (Figure 1A).
60 Curiously, depigmentation is a major trait contributing to the so-called "domestication
61 syndrome" observed in a variety of domesticated animals, but for reasons that are not
62 well-understood (Wilkins *et al.* 2014).

63 The genetic basis of pigmentation loss associated with *B. mori* domestication is
64 not yet known. Previous studies reported that melanin synthesis pathway genes *tyrosine*
65 *hydroxylase* (*TH*) and *aspartate decarboxylase* (*ADC*, also known as *black*) were
66 potentially targets of selection during silkworm domestication (Yu *et al.* 2011, Xiang *et*
67 *al.* 2018). Pigmentation patterning genes are also likely to be associated with the body
68 color differences between *B. mori* and *B. mandarina*. The wild-type *B. mori* larvae are
69 largely white but exhibit melanic spots (*i.e.* eye spots, crescent spots and star spots,
70 Figure 1A). *B. mandarina* larvae, on the other hand, are substantially darker, and
71 exhibit extensive dorsal pigment patterning that includes banding and spots (Figure 1A).

72 In *B. mori*, distinct alleles of the genetic locus, *p*, encode at least 15 different
73 larval markings such as spots, stripes, and banding (Yoda *et al.* 2014). The gene
74 underlying allelic variation at *p*, *apontic-like* (*apt-like*), encodes a transcription factor
75 that is likely to regulate the expression of melanin synthesis pathway genes such as
76 *yellow-y*, *ebony*, *TH*, *Dopa decarboxylase* (*DDC*) and *laccase 2* (Futahashi *et al.* 2008,
77 Yoda *et al.* 2014) (Figure 1B). While *apt-like* has been implicated in pigmentation
78 differences among *B. mori* strains, it's potential role in depigmentation of *B. mori* during
79 domestication is not known. A hybrid strain (semiconsomic T02), in which chromosome
80 2 of *B. mandarina* has been substituted into the genomic background of *B. mori*, exhibits
81 a phenotype similar to that of the *B. mori* allele *moricaud* (p^M) (Fujii *et al.* 2021). Since
82 *apt-like* resides on chromosome 2, dorsal pigmentation patterning on the larvae of *B.*
83 *mandarina* that is absent in *B. mori* was hypothesized to be controlled by the expression
84 of *apt-like* (Yoda *et al.* 2014, Fujii *et al.* 2021), although direct evidence is lacking.
85 The genetic analysis of domestication-associated loss of pigmentation in *B.*
86 *mori* has been challenging because deficiencies in candidate genes can result in lethality.
87 For example, in the fruit fly, *Drosophila melanogaster*, *TH*-deficient (*pale*) mutants die
88 at embryonic stage (Neckameyer and White 1993). In *B. mori*, *TH*-deficient mutants
89 (*sch lethal*, sch^l) and RNAi-mediated knockdowns of *TH* are both lethal at embryonic
90 stage (Liu *et al.* 2010). In addition, *apt* mutants in *D. melanogaster* die at embryonic
91 stage (Eulenberg and Schuh 1997, Gellon *et al.* 1997), and RNAi-mediated knockdown
92 of *apt-like* in *B. mori* embryos results in death before hatching (Yoda *et al.* 2014).

93 The issue of lethality is likely to continue to impede the genetic analysis of
94 pigmentation loss and other domestication-associated traits in *B. mori*. Recently, several
95 candidate genes associated with domestication in *B. mori* have been identified using
96 quantitative trait locus (QTL) mapping, including silk production (Li *et al.* 2017, Fang
97 *et al.* 2020), larval climbing ability (Wang, Lin, *et al.* 2020), and mimicry (Wang, Lin,
98 *et al.* 2020). In addition, a recent population genetic analysis identified 300 candidate
99 genes as targets of recent selection in *B. mori*, some of which are likely to be associated
100 with silk production and voltinism (Xiang *et al.* 2018). Despite these efforts, reverse
101 genetic approaches such as targeted gene knockouts and editing (Takasu *et al.* 2010,
102 2013, Wang *et al.* 2013), gene silencing (Quan *et al.* 2002), or transgenesis (Tamura *et*
103 *al.* 2000) will likely be required to fully understand the function of these candidate genes
104 and their potential contribution to domestication-associated traits in *B. mori*. Notably,
105 the functions of domestication candidate genes have been tested by reverse genetics
106 approaches in *B. mori* (Xiang *et al.* 2018) but these approaches, to our knowledge, have
107 not yet been implemented in *B. mandarina*.

108 Here we use CRISPR/Cas9-targeted knockouts of two candidate pigmentation
109 genes in two distinct contexts. First, we demonstrate the feasibility of CRISPR/Cas9-
110 targeted knockouts in *B. mandarina* by generating a homozygous *yellow-y* knockout
111 strain. Next, to circumvent the lethal effects of knocking out a second candidate gene,
112 *apt-like*, we use *allele-specific* CRISPR/Cas9-targeted knockouts in *B. mori* × *B.*
113 *mandarina* F_1 hybrids. The latter experiment also comprises a test (the "reciprocal

114 hemizygosity test", Steinmetz *et al.* 2002, Stern 2014) of the contribution of *apt-like*
115 evolution to the domestication-associated loss of pigmentation in *B. mori*.
116

117 Results and discussion

118 A CRISPR/Cas9-targeted knockout of *yellow-y* in *B. mandarina.*

119 To demonstrate the feasibility of CRISPR/Cas9-targeted knockouts in *B.*
120 *mandarina*, we focused on known pigmentation-related genes. *TH* is among the most
121 compelling candidate genes in the melanin synthesis pathway that seems likely to
122 underlie domestication-associated loss of pigmentation in *B. mori* (Yu *et al.* 2011, Xiang
123 *et al.* 2018). However, *TH* knockout mutants are predicted to be lethal (Neckameyer and
124 White 1993, Liu *et al.* 2010), rendering them difficult to study. Thus, we decided to
125 target *yellow-y* (Figure 1B), a melanin synthesis gene that is downstream of *TH* and
126 functions in melanin synthesis in wide range of insects including *B. mori* and other
127 Lepidoptera and is predicted to be non-essential (Futahashi *et al.* 2008, Zhang *et al.* 2017,
128 Chen *et al.* 2018, Matsuoka and Monteiro 2018, Liu *et al.* 2020, Wang, Huang, *et al.*
129 2020, Han *et al.* 2021, Shirai *et al.* 2021). After confirming the coding sequence
130 annotation (CDS) of the *B. mandarina yellow-y* gene, we designed a unique CRISPR-
131 RNA (crRNA) target site in exon 2 (Figure 2A, Table S1).

132 In *B. mori*, CRISPR/Cas9-targeted genome editing requires microinjection into
133 non-diapausing eggs (Kanda and Tamura 1994). We obtained non-diapausing eggs by
134 rearing *B. mandarina* larvae under 16 h-light/8 h-dark conditions (Kobayashi 1990). We
135 then injected a mixture of crRNA, trans-activating crRNA (tracrRNA), and Cas9 into

136 336 *B. mandarina* embryos. Among 16 hatched larvae (G_0 generation), nine grew to
137 adult moths (Table 1). We crossed six G_0 adults with wild-type moths and obtained
138 generation 1 (G_1) eggs. Using a heteroduplex mobility assay on the PCR products from
139 G_1 embryos, we confirmed that mutations were introduced at the target site in five of the
140 six G_1 broods (Table 1, Figure S1), showing that CRISPR/Cas9-induced mutations of
141 *yellow-y* were heritable. We then crossed G_1 siblings with each other and obtained
142 *yellow-y* homozygous knockout individuals carrying a five-nucleotide deletion followed
143 by a single-nucleotide substitution (Figure 2B), which results in a frame-shift and
144 premature stop codon. We designated this mutant allele *yellow-y*$^{\Delta 5}$ and used it for further
145 analyses.

146 *B. mandarina yellow-y*$^{\Delta 5}$ homozygotes hatched normally and their development
147 was comparable to that of wild-type individuals (Figure 3). In homozygous *yellow-y*$^{\Delta 5}$
148 neonate larvae, the larval integument and the head capsule are reddish brown instead of
149 the normal black (Figure 3A) and in final instar larvae, spots and dorsal pigmentation
150 patterns are lighter than that of the wild type (Figure 3B). Later in development, the
151 pupal integument of homozygous *yellow-y*$^{\Delta 5}$ mutants exhibits reddish color instead of
152 the normal black (Figure 3C), and the body and wing spot markings of *yellow-y*$^{\Delta 5}$ adult
153 moths are lighter than that of wild-type (Figure 3D). Further, the phenotypes of
154 heterozygous *+/yellow-y*$^{\Delta 5}$ individuals were comparable to that of wild type (data not
155 shown). Together, these observations suggest that, as observed in *B. mori* (Futahashi *et*
156 *al.* 2008), *yellow-y* is a non-essential gene contributing to melanin pigment synthesis in
157 *B. mandarina* and loss-of-function is recessive.

158 Comparative data from other species suggests that *yellow-y* functions
159 differently among lepidopteran species. For example, while *yellow-y* loss-of-function is
160 also found to be recessive in several other lepidopteran species (Liu *et al.* 2020, Wang,
161 Huang, *et al.* 2020, Han *et al.* 2021, Shirai *et al.* 2021), it is dominant in the black
162 cutworm, *Agrotis ipsilon* (Chen *et al.* 2018). Further, unlike *Bombyx*, *yellow-y*
163 knockouts in *Agrotis* are susceptible to dehydration (Chen *et al.* 2018) and mutants in
164 *Spodoptera* exhibit defects in body development, copulation, oviposition, and
165 hatchability (Liu *et al.* 2020, Han *et al.* 2021, Shirai *et al.* 2021). These observations
166 suggest that although the function of *yellow-y* in melanin synthesis is conserved,
167 additional *yellow-y* functions might be diverged among lepidopteran species. *yellow*
168 genes are a rapidly evolving gene family, and loss or duplication of some *yellow* genes
169 have been observed in Lepidoptera (Chen *et al.* 2018, Liu *et al.* 2020, Han *et al.* 2021,
170 Shirai *et al.* 2021). It is possible that functions of *yellow-y* outside of melanin synthesis
171 in *Bombyx* are compensated for by other *yellow* paralogs. In Lepidoptera, the function
172 of most *yellow* genes remained unknown except for *yellow-y* (Futahashi *et al.* 2008,
173 Zhang *et al.* 2017, Chen *et al.* 2018, Matsuoka and Monteiro 2018, Liu *et al.* 2020, Wang,
174 Huang, *et al.* 2020, Han *et al.* 2021, Shirai *et al.* 2021), *yellow-e* (Ito *et al.* 2010), *yellow-*
175 *d* (Zhang *et al.* 2017) and *yellow-h2/3* (Zhang *et al.* 2017). Further studies of *yellow*
176 genes including *yellow-y* in diverse insect species are required to understand the
177 functions and evolution of *yellow* paralogs.

178 While our results demonstrate the feasibility of genome editing in *B. mandarina*,
179 these experiments are challenging due to low post-injection hatchability rates (4.8% in

180 our experiment). A recent study showed that the hatchability of *B. mandarina* eggs is
181 generally lower than *B. mori* eggs, even under normal conditions (Zhu *et al.* 2019). To
182 compare the hatchability of *B. mori* and *B. mandarina* embryos after injection, we
183 injected three commonly used buffers (see Methods) and distilled water into embryos of
184 both species and compared hatching rates. We found that the post-injection hatchability
185 of *B. mandarina* embryos (0–8.3 %) is substantially lower than that of *B. mori* (22.9–
186 62.5 %) in all experimental conditions (Table 2), suggesting that *B. mandarina* embryos
187 are more sensitive to injection.
188
189 ***Allele-specific* knockouts of *apt-like* in interspecific F_1 hybrids.**
190 Knockouts of some pigmentation pathway genes, such as *apt-like* and *TH*, are
191 predicted to be lethal (Neckameyer and White 1993, Eulenberg and Schuh 1997, Gellon
192 *et al.* 1997, Liu *et al.* 2010, Yoda *et al.* 2014). Considering this obstacle to the study of
193 essential genes, together with the observation (above) of reduced hatchability of injected
194 *B. mandarina* embryos, we instead opted for the alternative strategy of injecting *B. mori*
195 × *B. mandarina* F_1 hybrids. Specifically, we conducted an *allele-specific* CRISPR/Cas9-
196 targeted knockout of *apt-like* in F_1 embryos (from crosses between *B. mori* females and
197 *B. mandarina* males). We reasoned that, because the components of these F_1 eggs is
198 derived from the *B. mori* mother, these embryos should have post-injection hatchability
199 similar to that of *B. mori*. We designed species-specific crRNA targeting *apt-like*, for
200 which the targeted protospacer adjacent motif (PAM) sequence is only present in either
201 *B. mori*- or *B. mandarina*-derived sequence (Methods, Figure 4, Table S1). For each

202 targeted allele, we injected a mixture of crRNA, tracrRNA and Cas9 into 48 F_1 embryos.
203 To confirm that mutations were specifically introduced into the targeted allele, we
204 extracted genomic DNA from adult legs and PCR-amplified the target sites. We then
205 carried out heteroduplex mobility assays using a microchip electrophoresis system (Ota
206 *et al.* 2013, Ansai *et al.* 2014), which show that various mutations were introduced into
207 the *apt-like* target sequence in both of the *allele-specific* knockout series (Figure S2).
208 The PCR products obtained from two representative individuals of each *allele-specific*
209 knockout series were then cloned and sequenced (Figure 4). The sequences of cloned
210 PCR products confirmed that various mutations were introduced into the target sites in
211 both knockout series, some of which cause frameshifts and associated premature stop
212 codons. All mutations detected in this experiment were specifically introduced only into
213 the targeted allele.

214 In addition to spots, normal F_1 larvae have a dark body with darker greyish
215 brown banding covering wide range of the dorsal surface, similar to the pattern for *B.*
216 *mandarina* (Figure 1A, Figure S3). As predicted, post-injection hatchability of F_1
217 embryos was high whether targeting the *B. mori* (60%) or the *B. mandarina* (66%)
218 allele. In the *apt-like* knockout series targeting the *B. mori* allele, all of the 26 larvae that
219 survived to the fifth instar stage exhibited normal body color (Table 3, Figure 5). In
220 contrast, for the *apt-like* knockout series targeting the *B. mandarina* allele, 24 of the 29
221 larvae that survived to the fifth instar stage exhibited white patches on dorsal
222 pigmentation pattern banding that varied in size (Table 3, Figure 5, Figure S4). This
223 result establishes a role for *apt-like* in body pigment formation and that the *B.*

224 *mandarina*-derived *apt-like* allele is dominant with respect to this trait. Notably,
225 however, the pigmentation of F_1 pupae and adult stages of the knockout series targeting
226 the *B. mori* or the *B. mandarina* allele both exhibited normal body color (data not shown).
227 Additionally, larval spots, which are also predicted to be controlled by *apt-like* (Yoda *et*
228 *al.* 2014), were also not affected the F_1 series targeting either the *B. mori* or the *B.*
229 *mandarina* allele (Figure 5, Figure S4). Since both wild-type ($+^p$) *B. mori* and *B.*
230 *mandarina* larvae exhibit spots (Figure 1A), we conclude that the *B. mori* or the *B.*
231 *mandarina*-derived *apt-like* alleles are both sufficient to direct the formation of larval
232 spots.

233 Our results have implications beyond merely confirming a role for *apt-like* in
234 *B. mandarina* larval pigmentation. The *allele-specific* knockouts of *apt-like* in F_1 hybrids
235 allows us to compare the phenotype of genetically identical hybrids that differ only at
236 the target locus (a framework called the "reciprocal hemizygosity test", Steinmetz *et al.*
237 2002, Stern 2014). As such, we can attribute the loss of pigmentation in the series
238 targeting the *B. mandarina* allele to evolution at the *apt-like* gene in *B. mori,* rather than
239 exclusively at a *trans*-acting factor. The Apt-like proteins of *B. mori* (p50T) and *B.*
240 *mandarina* (Sakado) differ by only one amino acid substitution: Alanine to Valine at
241 residue 188 (see DDBJ accession numbers LC706749 and LC706750). However, this
242 substitution is not observed in *B. mandarina* collected at different locations (see NCBI
243 accession numbers SRR6111377, SRR6111379, SRR6111381 and SRR6111382),
244 suggesting this is not a fixed amino acid difference between species. This implies that

245 evolution of an *apt-like cis*-regulatory element contributes to the observed phenotypic
246 difference between species.
247
248 **Conclusion**
249 Here we demonstrate the utility of CRISPR/cas9 genome editing in *B.*
250 *mandarina* and *B. mori* × *B. mandarina* F_1 hybrids to the study the function and
251 evolution of domestication-associated candidate genes. Focusing on two pigmentation-
252 related genes, we show that *apt-like* plays a role in larval body pigmentation patterning
253 (Figure 5), whereas *yellow-y* plays a more general role in pigmentation that is not pattern
254 dependent or specific to developmental life-stage (Figure 3B). These results are
255 consistent with the proposed roles of Apt-like as a transcription factor responsible for
256 larval color patterning, and Yellow-y as an enzyme in the melanin synthesis pathway
257 under control of Apt-like (Futahashi *et al.* 2008, Yoda *et al.* 2014). Further, using the
258 framework of the reciprocal hemizygosity test, we show that *apt-like* has evolved in *B.*
259 *mori* in a way that has specifically reduced larval body pigmentation, without affecting
260 the formation of larval spots or adult body pigmentation.
261 Despite being a powerful tool to study gene function and evolution, the *allele-*
262 *specific* knockout approach has several limitations. First, it requires sequence variants
263 distinguishing the parents of the F_1 hybrid that result in a PAM-site that is only present
264 in one of two species, limiting the potential to design *allele-specific* targets. However,
265 this limitation may be overcome by using Cas proteins that recognize different PAM-
266 sites (Leenay and Beisel 2017). For example, while the *Streptococcus pyogenes* Cas9

267 (*Sp*Cas9) protein has been used here, the Cas12a, which recognizes a distinct PAM
268 sequence, has also been implemented in *B. mori* (Dong *et al.* 2020). In addition, recent
269 studies have reported that *Sp*Cas9 can be modified to recognize alternative PAM
270 sequences (Kleinstiver *et al.* 2015, 2016). A second limitation is that, given the
271 mosacism of knockouts in G_0 individuals, one cannot exclude the possibility of false-
272 negative phenotyping results. The cleavage efficiency of the CRISPR/Cas9 system is
273 affected by several features, such as the sequences of PAM-distal and PAM-proximal
274 regions of the guide RNA, the genomic context of the targeted DNA, as well as GC-
275 content and secondary structure of the guide RNA (Liu *et al.* 2016). To minimize this
276 problem, one can screen a large number of G_0 individuals and confirm that mutations
277 were introduced with high efficiency (as in Figure 4 and Figure S2).

278 Despite these limitations, our results highlight several advantages of *allele-*
279 *specific* knockouts in the F_1 over knockouts in *B. mandarina*. First, our results show that
280 *B. mori* (female) × *B. mandarina* (male) F_1 embryos are substantially more tolerant to
281 injection compared to *B. mandarina*. Second, *allele-specific* knockouts in the F_1 permit
282 the study of essential genes (such as *apt-like*) at which knockouts are expected to be
283 homozygous lethal and recessive with respect to the phenotype. Finally, *allele-specific*
284 CRISPR/Cas9-targeted knockouts in F_1 hybrids have the added utility of identifying loci
285 that have diverged in function between *B. mori* and *B. mandarina*, and contributing to
286 domestication-related traits in *B. mori* using the framework of the reciprocal
287 hemizygosity test. Thus, our study showcases the multifaceted utility of *allele-specific*
288 knockouts in F_1 hybrids in the study of gene function and evolution.

289 ## Experimental procedures

290 **Insects**

291 The *B. mori* strain p50T, a single-paired descendant of individuals of strain p50
292 (a derivative from Daizo;
293 https://shigen.nig.ac.jp/silkwormbase/ViewStrainDetail.do?name=p50), is maintained
294 at our laboratory. The *B. mandarina* strain, Sakado, was originally collected in Sakado-
295 city, Saitama, Japan, in 1982. Since then, it has been maintained at our laboratory by
296 sib-mating. All larvae were reared on fresh mulberry leaves or artificial diet (SilkMate
297 PS, NOSAN) under continuous 12 h-light/12 h-dark conditions at 25 °C with the
298 exceptions described below. For injections in to *B. mandarina* embryos, we obtained
299 non-diapausing eggs by rearing *B. mandarina* larvae under continuous long-day
300 conditions (16 h-light/8 h-dark) at 25 °C (Kobayashi 1990). We then collected eggs in
301 crosses between emerged adults. To generate *B. mori* × *B. mandarina* F_1 hybrid
302 embryos for injection, we first incubated *B. mori* eggs at 15 ºC under continuous
303 darkness (Kogure 1933). The hatched larvae were then reared under continuous 16 h-
304 light/8 h-dark condition at 25 °C and females were crossed to *B. mandarina* males.

305

306 **Confirmation of the *yellow-y* and *apt-like* coding sequences in *B. mandarina*.**

307 Total RNA was extracted from the integument of fourth instar *B. mandarina*
308 larvae using TRIzol (Thermo Fisher Scientific). Complementary DNA (cDNA) was
309 reverse transcribed from total RNA using TaKaRa RNA PCR Kit (TaKaRa). Reverse
310 transcriptase-PCR was performed using KOD One polymerase (TOYOBO). PCR

311 products were cloned into pGEM-T Easy Vector (Promega) and Sanger-sequenced
312 using the FASMAC sequencing service (Kanagawa, Japan).
313
314 **Knockout of *yellow-y* in *B. mandarina***
315 An unique crRNA target sequence in the *B. mori* genome was selected using
316 CRISPRdirect (https://crispr.dbcls.jp) (Table S1) (Naito *et al.* 2015). The uniqueness
317 of the target sequence in the *B. mandarina* genome was then confirmed by performing
318 blastn at SilkBase (http://silkbase.ab.a.u-tokyo.ac.jp). A mixture of crRNA, tracrRNA
319 and Cas9 Nuclease protein NLS (600 ng/μL; NIPPON GENE) in injection buffer (100
320 mM KOAc, 2 mM $Mg(OAc)_2$, 30 mM HEPES-KOH; pH 7.4) was injected into each
321 embryo within 3 h after oviposition (Yamaguchi *et al.* 2011).
322 The injected embryos (G_0 generation) were incubated at 25 °C in a humidified
323 Petri dish until hatching. Adult G_0 moths were crossed with wild-type *B. mandarina*,
324 and G_1 eggs were obtained. To detect heritable CRISPR/Cas9-induced mutations, ten
325 G_1 eggs were collected into one tube, and genomic DNA was prepared using the
326 HotSHOT method (Truett *et al.* 2000). The region containing the target site of *yellow-y*
327 crRNA was PCR-amplified using KOD One polymerase (TOYOBO). Mutations at the
328 target site were detected by heteroduplex mobility assay using the MultiNA microchip
329 electrophoresis system (SHIMAZU) with the DNA-500 reagent kit (Ota *et al.* 2013,
330 Ansai *et al.* 2014).
331 Adult G_1 moths from broods with heritable CRISPR/Cas9-induced mutations were
332 crossed with each other to obtain homozygous knockout mutants (G_2). To confirm

333 CRISPR/Cas9-induced mutations, we prepared genomic DNA, PCR-amplified the
334 target region and detected mutations as described above using G_2 adult moths. To
335 determine the precise nature of insertions, deletions and substitutions, PCR products
336 obtained from G_2 individuals were directly Sanger-sequenced using the FASMAC
337 sequencing service (Kanagawa, Japan).
338
339 **Comparison of post-injection hatchability.**
340 Three commonly used buffers (injection buffer 1 (Yamaguchi *et al.* 2011),
341 injection buffer 2 (Tamura *et al.* 2000), PBS buffer) and distilled water into embryos of
342 *B. mori* or *B. mandarina* within 3 h after oviposition. The injected embryos were
343 incubated at 25 °C in a humidified Petri dish until hatching.
344
345 ***Allele-specific* gene knockouts in F_1 hybrids.**
346 A PAM sequence is necessary for target recognition and following DNA
347 cleavage in CRISPR/Cas system (Hsu *et al.* 2013, Anders *et al.* 2014), and *Sp*Cas9,
348 which we used in this study, recognizes 5'-NGG-3' as PAM. We specifically targeted
349 *Sp*Cas9 PAM-sites that differed in sequence between *B. mori* and *B. mandarina* (Figure
350 4, Table S1), allowing *allele-specific* DNA cleavage and gene knockout (Courtney *et*
351 *al.* 2015, Christie *et al.* 2017). A mixture of crRNA, tracrRNA and Cas9 was injected
352 to F_1 embryos as described above. To evaluate CRISPR/Cas9 cleavage efficiency, we
353 extracted genomic DNA from G_0 adult legs and PCR-amplified the target region as

354 described above. PCR products were cloned into pGEM-T Easy Vector and Sanger-
355 sequenced using an ABI3130xl genetic analyzer (Applied Biosystems).
356

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364

365 **Data Availability**

366 Full-length coding sequences of *B. mori yellow-y*, *B. mandarina yellow-y*, *B.*
367 *mori apt-like* and *B. mandarina apt-like* are available on the DDBJ under accession
368 numbers of LC706747, LC706748, LC706749 and LC706750, respectively.
369

370 **Author contributions**

371 KT, PA and TK designed the study. KT performed most of the experiments. KT
372 wrote the manuscript with intellectual input from TK and PA. All authors edited and
373 approved the final version of the manuscript and agree to be accountable for all aspects
374 of the work.
375

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540 **Figures and Tables**

541

542 **Table 1.** Efficiency of CRISPR/Cas9-targeted knockout in *B. mandarina* targeting

543 *yellow-y*.

# eggs injected	# hatched larvae	# adults	# G_1 broods	# G_1 broods carrying mutant alleles
336	16	9	6	5

544

545

546 **Table 2.** The hatchability of *B. mori* and *B. mandarina* embryos injected with an

547 injection buffer 1 (Yamaguchi *et al.* 2011), injection buffer 2 (Tamura *et al.* 2000), PBS

548 buffer and distilled water.

Treatment	Composition	*B. mori*		*B. mandarina*	
		# injected eggs	# hatched larvae	# injected eggs	# hatched larvae
Injection buffer 1	100 mM KOAc, 2 mM $Mg(OAc)_2$, 30 mM HEPES-KOH; pH 7.4	48	11	48	0
Injection buffer 2	0.5 mM phosphate buffer (pH 7.0), 5 mM KCl	48	30	48	4
PBS buffer	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76mM KH_2PO_4; pH 7.4	48	23	48	1
Distilled water	–	48	17	48	2

549

550 **Table 3.** Efficiency of *allele-specific* knockouts of *apt-like* in F_1 hybrids.

Targeted allele	# eggs injected	# hatched larvae	# 5th instar larvae	# mosaic 5th instar larvae	# adults (male/female)
B. mori	48	29	26	0	26 (16/10)
B. mandarina	48	32	29	24	29 (18/11)

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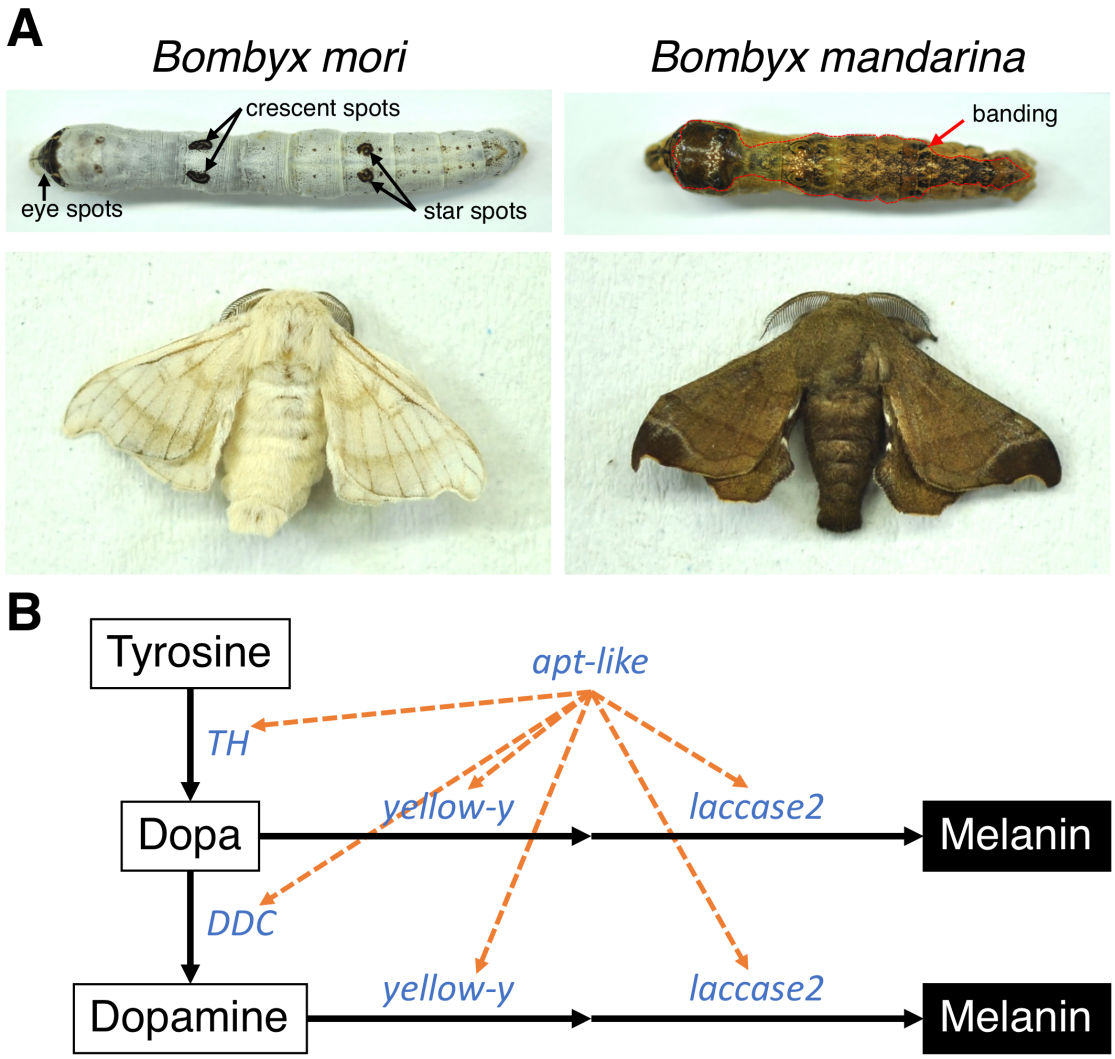


552

553 **Figure 1.** (A) Larvae (top) and adult males (bottom) of *B. mori* (left) and *B. mandarina*

554 (right). Black arrows indicate larval spot markings (eye spots, crescent spots and star

555 spots) and red dotted lines delimit the larval dorsal pigment patterning ("banding").

556 The names of spots follow the nomenclature of Yoda *et al.* (2014). (B) The proposed

557 melanin biosynthesis pathway in *Bombyx* larvae largely adapted from Futahashi *et al.*

558 (2008) and Yoda *et al.* (2014). Orange dashed arrows indicate presumed regulation of

559 genes by the transcription factor *apt-like*.

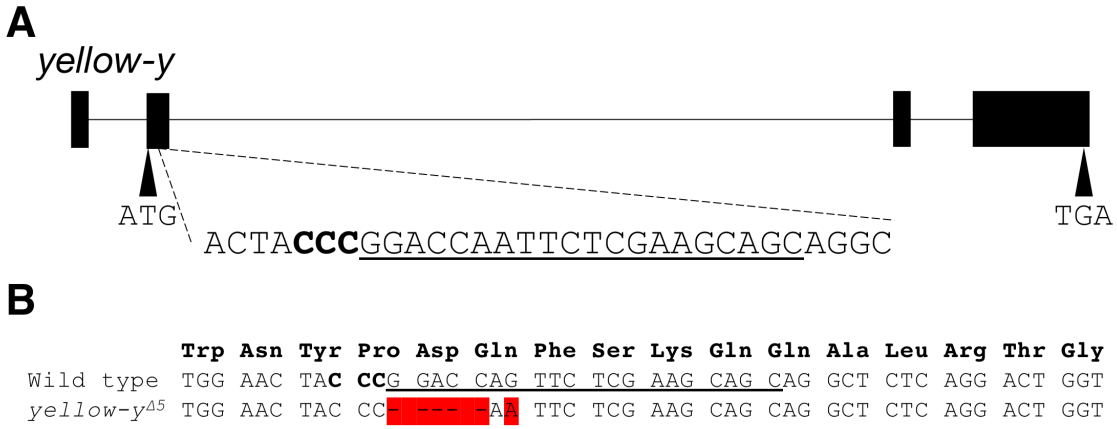


Figure 2. The nature of the lesion in the *yellow-y* knockout (*yellow-y*$^{\Delta 5}$) in *B. mandarina*. (A) Gene structure of *yellow-y* and the selected crRNA target site. The target sequence is underlined and the protospacer adjacent motif (PAM) site is shown in bold letters. (B) Alignment of the *yellow-y* gene sequences surrounding the crRNA target site from wild type, and G_2 *B. mandarina* individuals that are homozygous for a 5-base pair deletion and associated single nucleotide mutation. The target sequence is underlined, and the PAM site (CCN) is indicated in bold letters.

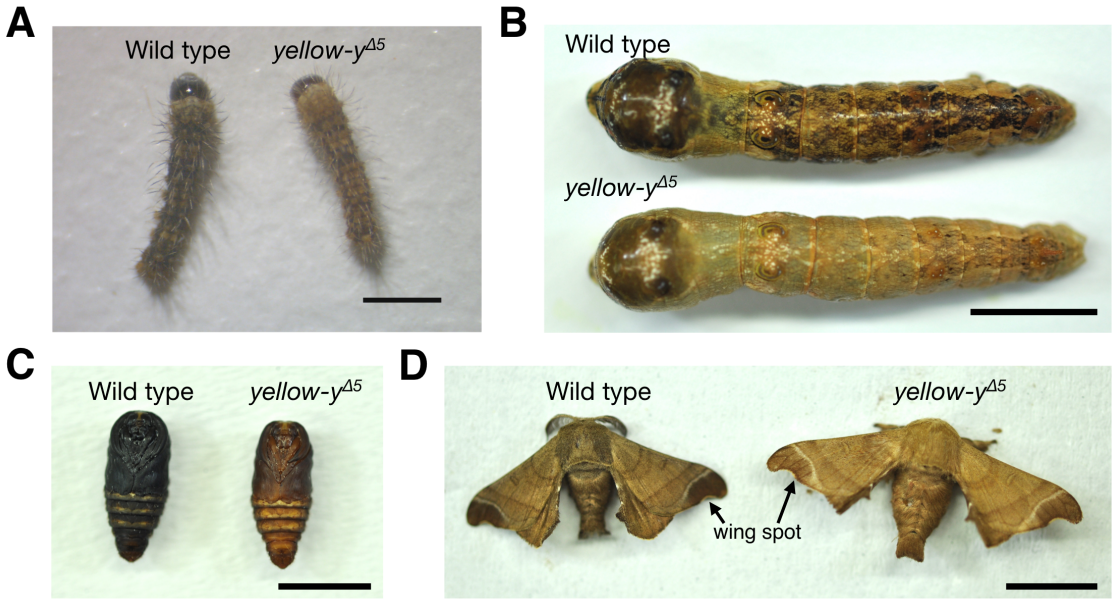


Figure 3. The phenotypes of wild-type and *yellow-y* knockout mutants of *B. mandarina*. Representative (A) neonate larvae, (B) final-instar larvae, (C) male pupae and (D) adult male moths of wild-type (left or top) and *yellow-y*$^{\Delta 5}$ (right or bottom) *B. mandarina*. Arrows indicate wing spot markings in adult moths. Scale bars: 1 mm (A) or 1 cm (B-D).

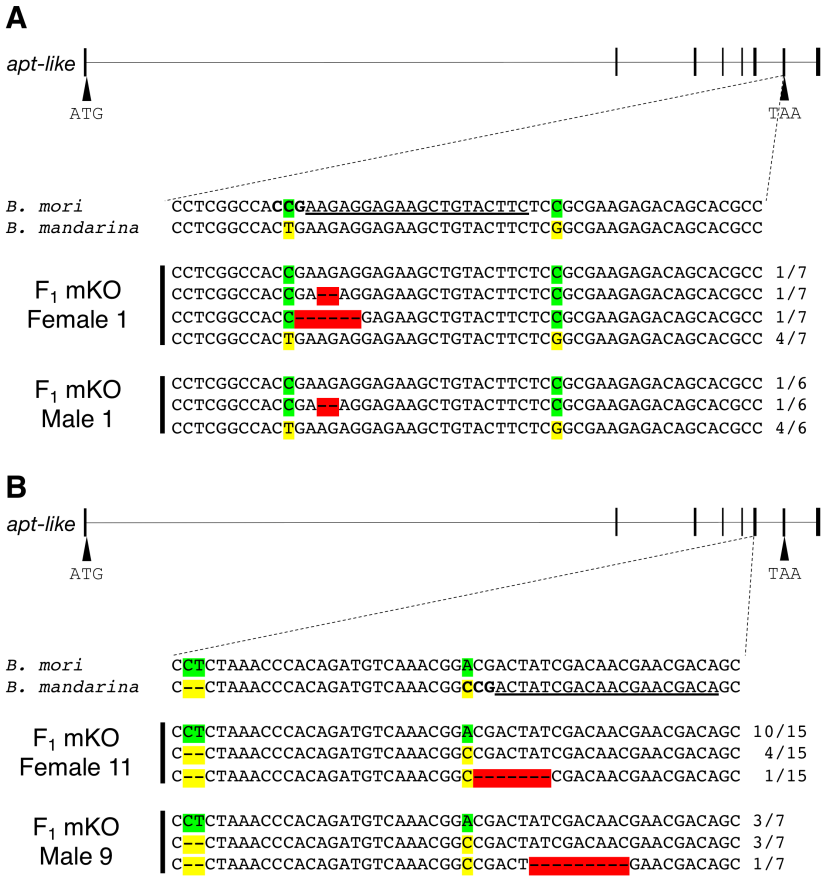
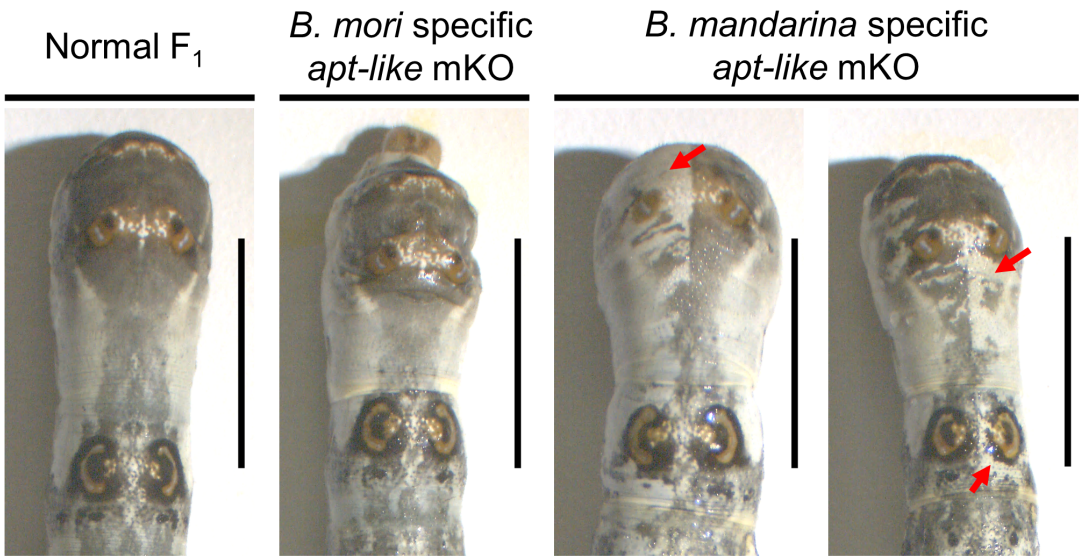


Figure 4. Mutations introduced by *allele-specific apt-like* mosaic knockout (mKO) in F_1 hybrids. Annotation and inset alignments of the *apt-like* gene in both species with selected crRNA target sites whose PAM sequences are only present in (A) *B. mori* or (B) *B. mandarina*. Two *allele-specific* knockout individuals were sequenced for each target. The target sequences are underlined, and the PAM sequences are shown in bold letters. *B. mori* specific SNPs and *B. mandarina* specific single nucleotide variants are highlighted with green and yellow shadings, respectively. Mutations introduced by CRISPR/Cas9 system are highlighted with red shading. Numbers on the right edge indicate the numbers of the clones identified among all cloned and sequenced PCR products.



587

588 **Figure 5.** Representative fifth instar larvae of normal F_1 (left), *allele-specific apt-like*

589 mKOs targeting the *B. mori* allele (middle) and the *B. mandarina* allele (right). Red

590 arrows indicate ectopic white (depigmented) regions. Scale bars: 1 cm.

591

592 **Table S1** PCR primers and RNA sequences used in this study.

Primer Name	Sequence	Usage
PCR primers		
yellow-y_cloning_F02	AAGCTGCCGCGAACATAG	RT-PCR and CDS cloning
yellow-y_cloning_R02	TGCAATAGAAAGCCTCACATTT	RT-PCR and CDS cloning
apt-like_cloning_F	GAAAATAAGAGCCCACGCACT	RT-PCR and CDS cloning
apt-like_cloning_R	ACTTGAATCCGCTTTTGACG	RT-PCR and CDS cloning
yellow-y_mid_F	CGTCTTTGGGTATTAGACGTTGG	CDS cloning
yellow_y_check_F	GTACATACCTGAGCGCCACCT	Genomic PCR for mutation detection and sequencing in CRISPR/Cas9-targeted gene knockout
yellow_y_check_R	CAGCAACGATAAAGCTCCAAG	Genomic PCR for mutation detection and sequencing in CRISPR/Cas9-targeted gene knockout
Bmand_apt-like_F03	GAGGTTTTGTGTGGCGAGATG	Genomic PCR for mutation detection and sequencing in CRISPR/Cas9-targeted gene knockout
Bmand_apt-like_R02	AGGAAGTTCTGCTGGAGTCTG	Genomic PCR for mutation detection and sequencing in CRISPR/Cas9-targeted gene knockout
Bmori_apt-like_F01	CTGGACGATCACATAAAGCAC	Genomic PCR for mutation detection and sequencing in CRISPR/Cas9-targeted gene knockout
Bmori_apt-like_R01	TCTCAACTCAATGAGGAACAGC	Genomic PCR for mutation detection and sequencing in CRISPR/Cas9-targeted gene knockout
RNAs		
yellow-y_crRNA_1	GCUGCUUCGAGAAUUGGUCCGUUUUAGAGCUAUGCUGUUUUG	CRISPR/Cas9-targeted gene knockout
Bmori_apt-like_specific_crRNA	GAAGUACAGCUUCUCCUCUUGUUUUAGAGCUAUGCUGUUUUG	CRISPR/Cas9-targeted gene knockout
Bmand_apt-like_specific_crRNA	UGUCGUUCGUUGUCGAUAGUGUUUUAGAGCUAUGCUGUUUUG	CRISPR/Cas9-targeted gene knockout
tracrRNA	AAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	CRISPR/Cas9-targeted gene knockout

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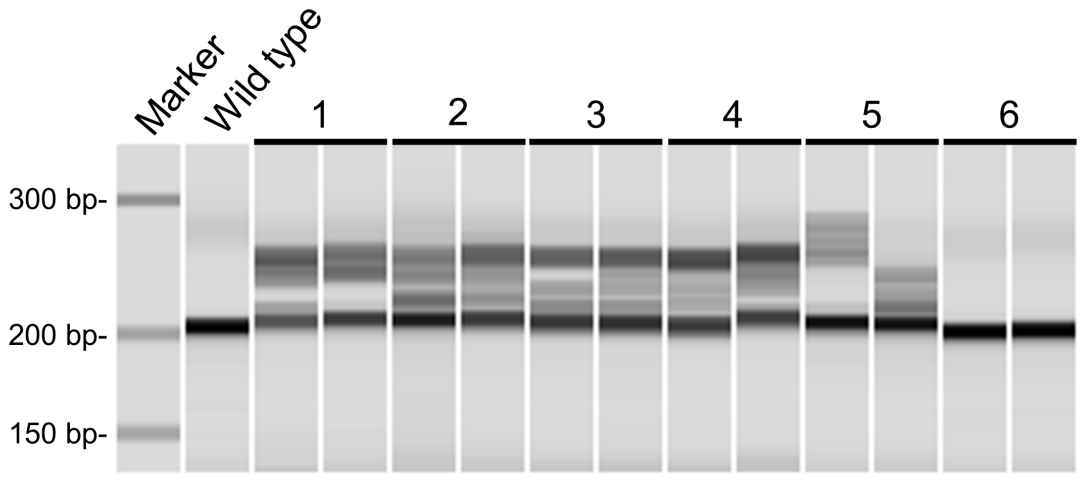


Figure S1. Detection of mutations at the *yellow-y* crRNA target site using a heteroduplex mobility shift assay. Two intervals were prepared for each G_1 brood. Ten eggs were collected into one tube, and genomic DNA was prepared. The region containing the target site of *yellow-y* crRNA was PCR-amplified. Multiple heteroduplex bands caused by insertion/deletion mismatches were observed indicating the presence of DNA lesions (insertions/deletions and/or associated nucleotide variants).

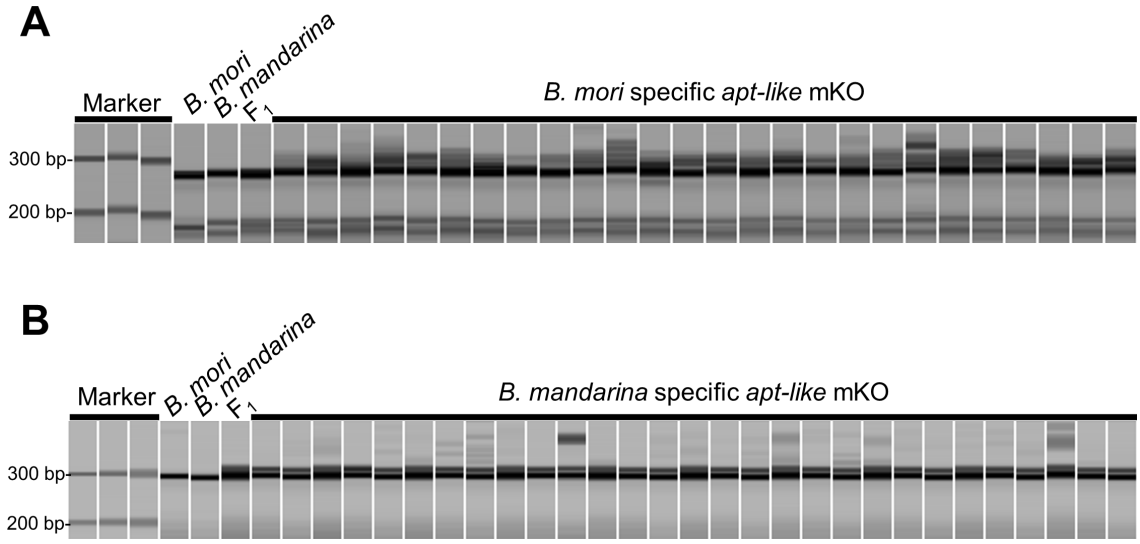


Figure S2. Detection of mutations at *apt-like* crRNA target sites using a heteroduplex mobility shift assay. The region containing the target site of (A) *B. mori* specific *apt-like* crRNA and (B) *B. mandarina* specific *apt-like* crRNA was PCR-amplified using DNA prepared from G_0 adults' legs. Multiple heteroduplex bands caused by insertion/deletion mismatches and associated nucleotide variants were observed in G_0 mosaics.

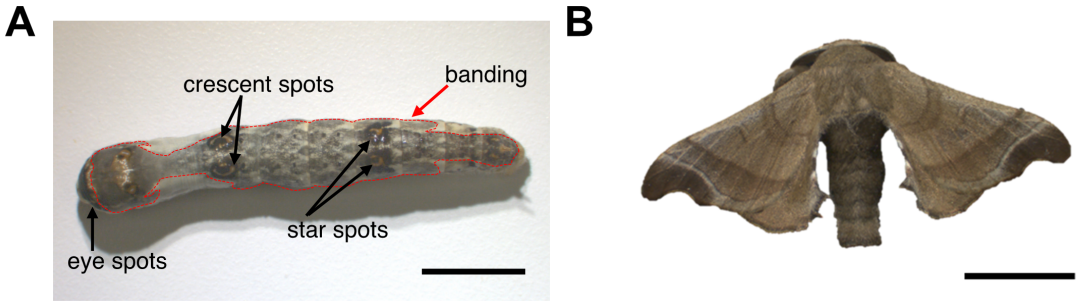
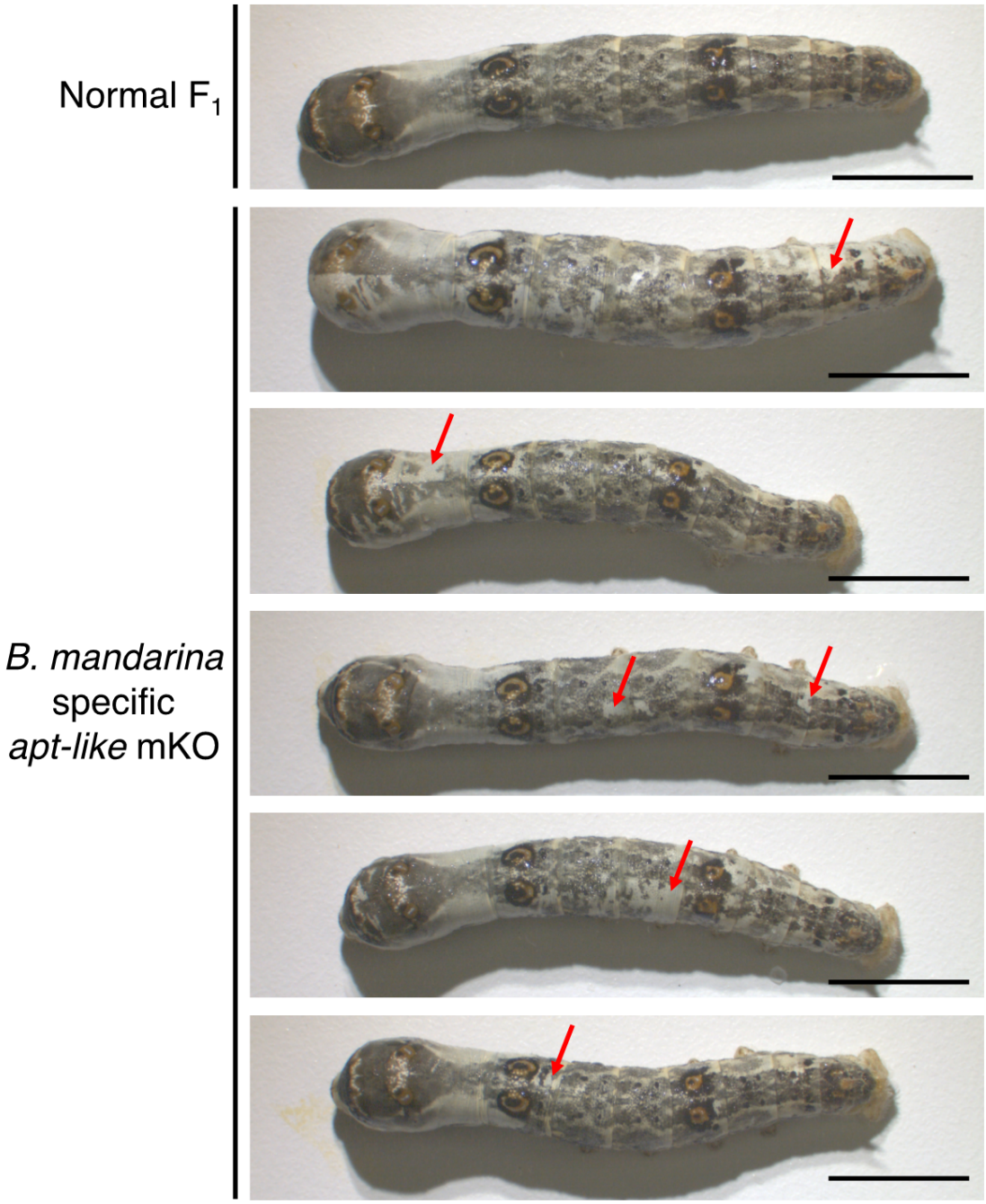


Figure S3. A representative (A) fifth instar larva and (B) adult male moth of normal F_1 (*B mori* female × *B. mandarina* male). Black arrows indicate larval spot markings and red dotted lines outline the banding. Normal F_1 larvae have a dark body with darker greyish brown banding covering wide range of the dorsal surface similar to *B. mandarina*. The names of spots were referred to Yoda *et al.* (2014). Scale bars: 1 cm.



620

621 **Figure S4.** Fifth instar larvae of normal F_1 and *B. mandarina* specific *apt-like* mKO.

622 Red arrows indicate ectopic white (depigmented) regions. Crescent and star spots were

623 not depigmented in all larvae. Scale bars: 1 cm.