

**Title**

**Repeated inversions at the *pannier* intron drive diversification of intraspecific colour**

**patterns of ladybird beetles**

1    **Abstract**

2                   How genetic information is modified to generate phenotypic variation within a  
3 species is one of the central questions in evolutionary biology. Here we focus on the striking  
4 intraspecific diversity of more than 200 aposematic elytral (forewing) colour patterns of the  
5 multicoloured Asian ladybird beetle, *Harmonia axyridis*, which is regulated by a tightly  
6 linked genetic locus *h*. Our loss-of-function analyses, genetic association studies, *de novo*  
7 genome assemblies, and gene expression data reveal that the GATA transcription factor gene  
8 *pannier* is the major regulatory gene located at the *h* locus, and suggest that repeated  
9 inversions and cis-regulatory modifications at *pannier* led to the expansion of colour pattern  
10 variation in *H. axyridis*. Moreover, we show that the colour patterning function of *pannier* is  
11 conserved in the seven spotted ladybird beetle, *Coccinella septempunctata*, suggesting that *H.*

12 *axyridis*' extraordinary intra-specific variation may have arisen from ancient modifications in

13 a conserved elytral colour patterning mechanisms in ladybird beetles.

14 **Main text**

15           There are approximately 6,000 ladybird beetle species described worldwide<sup>1</sup>.

16 Charismatic and popular, ladybird beetles are famous for the red and black spot patterns on

17 their elytra (forewings), thought to be a warning signal to predators that they store bitter

18 alkaloids in their body fluids<sup>2,3</sup> and are unpalatable. This red/black warning signal is shared

19 among many ladybird beetle species, and provides a model for colour pattern mimicry by

20 other insect orders. While most ladybird beetle species have only a single spot pattern, a few

21 display remarkable intraspecific diversities, such as the multicoloured Asian ladybird beetle,

22 *Harmonia axyridis*, which exhibits more than 200 different elytral colour forms (Fig.1a).

23 This striking intraspecific variation prompted us to investigate its genetic and evolutionary

24 basis.

25           The first predictions regarding the genetics underlying the highly diverse elytral  
26 colour patterns of *H. axyridis* were made by the evolutionary biologist, Theodosius  
27 Dobzhansky based on his comprehensive classification of specimens collected from various  
28 regions in Asia<sup>4</sup>. Successive genetic analyses<sup>5-7</sup> revealed that many of these colour patterns  
29 are actually regulated by a tightly linked genetic locus, *h*, which segregates either as a single  
30 gene, or as strongly linked pseudoallelic genes (a super-gene<sup>8, 9</sup>) (Fig.1b, c). The elytral  
31 colour patterns are assumed to be formed by the superposition of combinations of two of the  
32 four major allelic patterns and dozens of minor allelic colour patterns (more than 20 different  
33 allelic patterns in total). The major allelic patterns cover more than 95% of colour patterns in  
34 the natural population<sup>4</sup>. In the elytral regions where the different colour elements are  
35 overlapped in heterozygotes, black colour elements are invariably dominant against red

36 colour elements (mosaic dominance<sup>10</sup>). Whether all of the supposed alleles linked to the *h*  
37 locus correspond to a single gene or multiple genes is yet unknown. Elucidating the DNA  
38 structure and the mechanisms underlying the evolution of this tightly linked genetic locus  
39 that encodes such a strikingly diverse intraspecific colour pattern polymorphism would  
40 provide a case-study that bears upon a major evolutionary-developmental biology question;  
41 how does morphology evolve? Here we show that the gene *pannier* is responsible for  
42 controlling the major four elytral colour patterns of *H. axyridis*. Moreover, we illustrate how  
43 modification to this ancient colour-patterning gene likely contributed to an explosive  
44 diversification of colour forms.

45

46 **Figure 1. Intraspecific genetic polymorphisms of elytral colour patterns in *Harmonia***  
47 ***axyridis*.** **a**, Highly diverse elytral colour patterns of *H. axyridis*. **b**, Four major alleles of the

48 elytral colour patterns.  $h^C$ , *conspicua*;  $h^{Sp}$ , *spectabilis*;  $h^A$ , *axyridis*;  $h$ , *succinea*. **c**, An

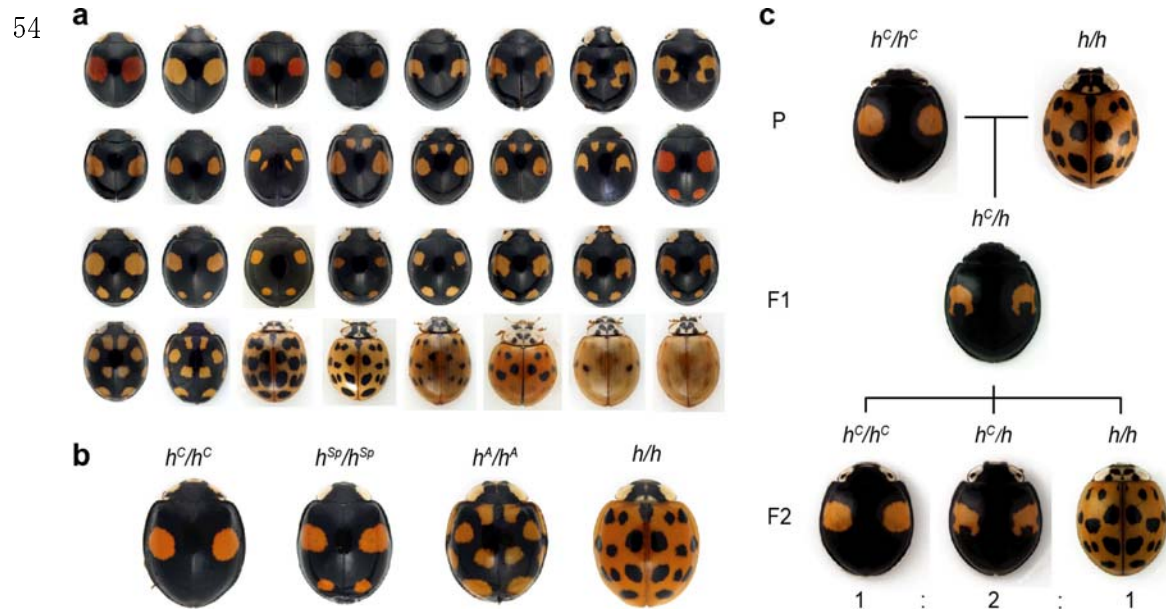
49 example of inheritance of elytral colour forms. When  $h^C/h^C$  and  $h/h$  are crossed (P), all F1

50 progenies show the colour pattern of  $h^C/h$ . Note the small black spots within the red spots.

51 When the F1 heterozygotes are sib-crossed, F2 progeny show three phenotypes ( $h^C/h^C$ ,  $h^C/h$

52 and  $h/h$ ) at the 1:2:1 ratio predicted for Mendelian segregation of a single locus. Inheritance

53 of any combination of colour patterns follows this segregation pattern.



55 **Results**

56 To identify the gene regulating elytral colour pattern formation of *H. axyridis*, we  
57 first investigated the pigmentation processes during development. In the developing pupal  
58 elytra, red pigment (carotenoids<sup>11</sup>) was accumulated in the future red-pigmented regions (Fig.  
59 2a pharate adult elytron). Red pigmentation occurred only in the thick ventral epidermal cells  
60 of the two layers of the elytral epidermis (Fig. 2b; 2c, Red), and started at 80 hours after  
61 pupation (80 h AP). Black pigmentation (melanin accumulation<sup>11</sup>) occurred only in the dorsal  
62 cuticle of black-pigmented regions (Fig. 2d), and started approximately 2 h after eclosion.  
63 Although pharate adult elytra are not black, we detected a strong upregulation of enzymatic  
64 activity related to melanin synthesis<sup>12</sup> in the nascent dorsal cuticle in the future black regions  
65 from 80 h AP (Fig. 2a, lower panels; Fig. 2c, black; Supplementary Figure 1). Every



66 black-pigmented region was deployed complementary to the red regions. Therefore, we

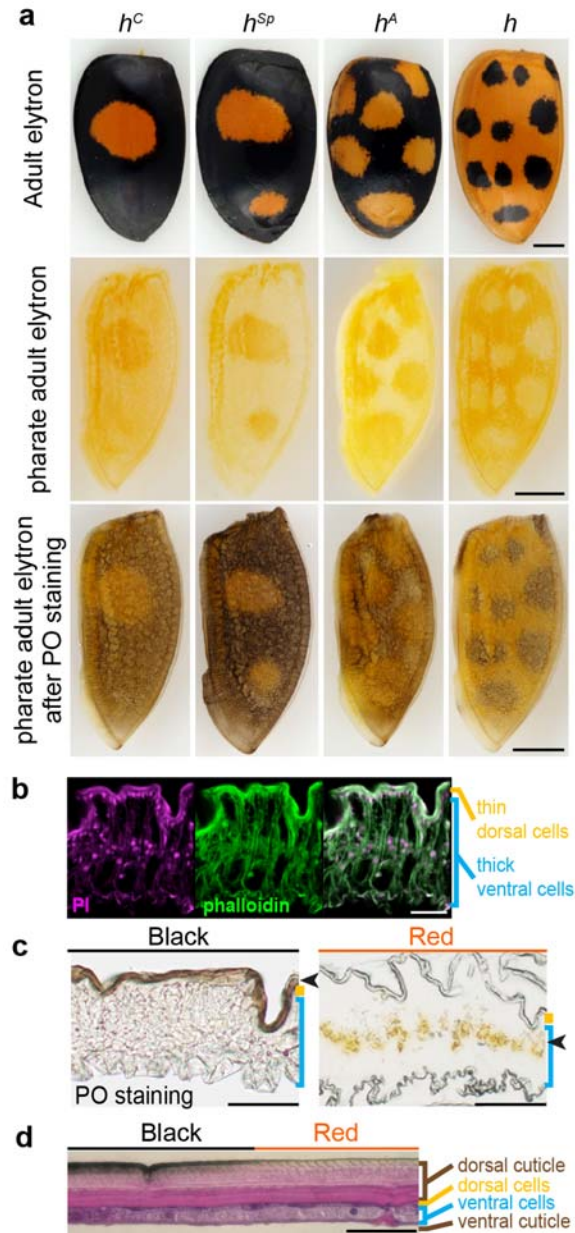
67 concluded that the developmental programmes for both red and black pigmentation started

68 around 80 h AP.

69

70 **Figure 2 | Developmental programmes for elytral pigmentation initiate at the late**  
 71 **pupal stage in *H. axyridis*.** **a**, Adult elytral colour patterns (upper panels), and localisation of

77

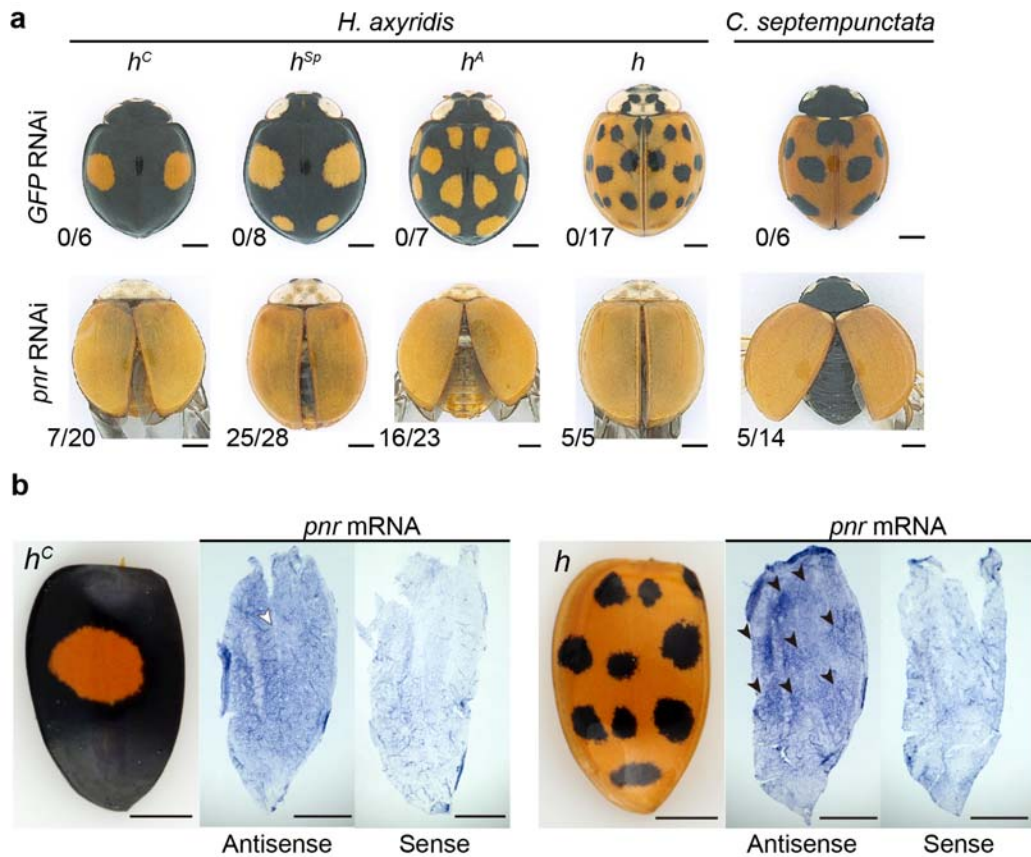


78           We hypothesised that some of the conserved genes essential for insect wing/body  
79 wall patterning<sup>13-18</sup> are recruited to regulate these elytral pigmentation processes, and tested  
80 this possibility using larval RNAi<sup>19</sup>. We performed small-scale candidate screening focusing  
81 on genes involved in wing/body wall patterning (Supplementary Table 1), and found that the  
82 *Harmonia* ortholog of *Drosophila pannier*, which encodes a GATA transcription factor<sup>20</sup>, is  
83 essential for formation of all of the black-pigmented regions in the elytra. For all four major *h*  
84 allele backgrounds, larval RNAi targeting *pannier* resulted in complete loss of black colour  
85 elements and alternative emergence of red colour elements in the elytra (Fig. 3a, *H. axyridis*),  
86 indicating that *pannier* is essential for inducing black pigmentation in dorsal elytral cells and  
87 suppressing red pigmentation in ventral elytral cells. This result was unexpected because  
88 *pannier* is not essential for wing blade patterning in *Drosophila*, but rather essential for

89 patterning of the dorsal body plate attached to the wings (notum)<sup>21-23</sup>.

90

91 **Figure 3 | The pattern of *pannier* expression foreshadowing the adult elytral colour**  
92 **pattern is necessary for switching red/black pigmentation processes in ladybird elytra.**  
93 **a.** The adult phenotypes of RNAi treatments targeting *GFP* (negative controls, *GFP* RNAi)  
94 and *pannier* (*pnr* RNAi) in *H. axyridis* ( $h^C$ ,  $h^{Sp}$ ,  $h^A$ ,  $h$ ) and *C. septempunctata*. Scores in the  
95 lower left corners indicate penetrance of the loss-of-pattern phenotype in surviving animals.  
96 **b.** The pattern of *pannier* expression (*pnr*) in the dorsal elytral epidermal cells immediately  
97 before or after pigmentation (76–84 h AP). Left panels indicate the corresponding adult  
98 elytral phenotypes ( $h^C$  and  $h$ ). White arrowhead, the region with a weak signal. Black  
99 arrowheads, the regions with intense signals. Scale bars, 1 mm.



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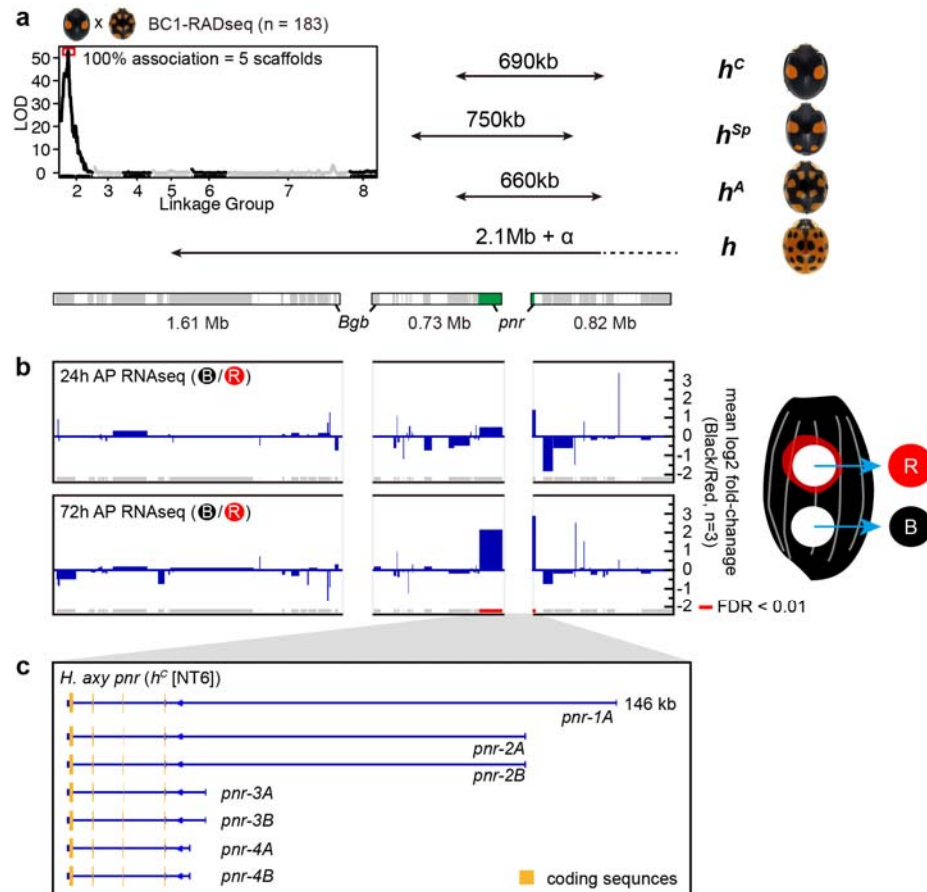
101 *pannier* mRNA was upregulated from 48 h AP to 96 h AP in elytra, and preferentially in  
102 black regions ( $h^C$ , Supplementary Figure 2). Immediately before or after 80 h AP (start of the  
103 pigmentation programme, 76–84h AP), *pannier* seemingly showed higher expression in the  
104 future black regions in the dorsal elytral epidermis (Fig. 3b). These data suggest that  
105 region-specific upregulation of *pannier* during the pupal stage regulates black pigmentation  
106 in the ladybird beetle's dorsal elytral cells, and that regions of expression differ among the  
107 major *h* alleles to form different black patterns in *H. axyridis*.

108           These data led us to test whether *pannier* is associated with the classically  
109 identified locus *h*, which regulates elytral colour patterns. To identify DNA sequences near  
110 the *h* locus, we assembled *de novo* genome sequences (assembly version 1: 423 Mb; contig  
111 N50, 63.5 kb; scaffold N50, 1.6 Mb), and performed a genetic association study using the

112 strains with different *h* alleles. We obtained the scaffold containing *pannier* and two  
113 additional adjacent scaffolds based on the truncated gene structures at the scaffold ends (Fig.  
114 4a, *Bgb* and *pnr*). Restriction-site Associated DNA Sequencing (RAD-seq) analysis of  
115 backcrossed progenies (BC1,  $h^A \times h^C$  F0 cross, n = 183) revealed that these three scaffolds are  
116 included in the five scaffolds that showed complete association with colour patterns (Fig. 4a,  
117 the upper left panel). In addition, genotyping of F2 individuals from two other independent  
118 genetic sib-crosses ( $h^C \times h$  F0 cross [n = 80] and  $h^A \times h^{Sp}$  F0 cross [n = 273]) indicated that the  
119 *pannier* locus is included in the relevant regions of all of the major four *h* alleles ( $h^C$ , 690 kb;  
120  $h^{Sp}$ , 750 kb;  $h^A$ , 660 kb; *h*, >2.1 Mb) (Fig. 4a, Supplementary Table 2).

121

122 **Figure 4 | *pannier* is the major elytral colour patterning gene located at the *h***  
 123 **locus. a,** Genetic association study of the *h* locus. Upper left panel, LOD plot for 4,419 RAD  
 124 tags deduced from genotyping BC1 progeny from the  $h^C-h^A$  F0 cross. RAD tags showing  
 125 segregation patterns of markers located on the X chromosome were excluded from the  
 126 analysis. The LOD score peaked at RAD tags in Linkage Group 2. RAD tags with complete  
 127 association with elytral colour patterns corresponded to 5 genomic scaffolds including the  
 128 three scaffolds adjacent to the *pannier* locus (lower bars). Upper right panel, the candidate  
 129 genomic regions responsible for the major four *h* alleles. Lower bars, the three genomic  
 130 scaffolds adjacent to the *pannier* locus. Grey, genes predicted from RNA-seq. Green, the  
 131 *pannier* locus. Contiguity of the scaffolds was predicted based on the truncated genes at the  
 132 end of scaffolds (*Bgb* and *pnr* [*pannier*]). Arrows indicate the respective candidate genomic  
 133 regions responsible for each allele. *pannier* is included in all four relevant regions. **b,** Fold  
 134 changes of gene expression in the presumptive red and black elytral epidermis before  
 135 pigmentation around the candidate genomic region responsible for *h*. Grey and red bars on  
 136 the bottom indicate predicted genes. Red, FDR < 0.01. Only *pannier* was significantly  
 137 upregulated in this region. The samples for RNA-seq were collected as depicted in the right  
 138 panel in  $h^C$  background. **c,** Gene structures of *pannier* in *H. axyridis*. 1A isoform cDNA was  
 139 cloned by rapid amplification of cDNA ends (RACE). 2A–4B isoforms were predicted from  
 140 RNA-seq analysis. *pannier* has at least 4 transcription start sites. Coding sequences are  
 141 located from exon 2 to exon 5 (yellow). There are two alternative exons at exon 3, one  
 142 encoding one of the two zinc finger domains of Pannier (A isoforms), and the other skipping  
 143 that zinc finger domain (B isoforms).  
 144



145



146 To test contiguity of these three scaffolds, we re-assembled the genome using a novel  
147 genome assembler (Platanus2), and additional *de novo* genomic assemblies of  $h^C$ ,  $h^A$  and  $h$   
148 alleles using linked-read and long read sequencing platforms (10x Genomics Chromium  
149 system; PacBio system). We obtained longer genomic scaffolds including the three described  
150 above ( $h^C$ , 3.13 Mb/2.74 Mb;  $h^A$ , 1.42 + 1.61 Mb;  $h$ , 2.79 Mb, Supplementary Table 3) and  
151 the genotyping markers showing complete association with colour patterns and incomplete  
152 association at both ends ( $h^C$  and  $h$ ) or one end ( $h^A$ ) of each scaffold (Supplementary Figures 3,  
153 4a–c). These data support the result of our genetic association studies.

154 To further delimit the candidate genes associated with the elytral colour patterns,  
155 we performed RNA-seq analysis using epidermal tissues isolated from the developing red or  
156 black regions before pigmentation in the  $h^C$  genetic background (Fig. 4b, 24 h & 72 h AP

157 RNA-seq). We found that *pannier* was the only gene statistically significantly upregulated in  
158 the developing black region compared to the red region at 72 h AP within the *h* locus  
159 candidate region (Fig. 4b, red bars, False discovery rate [FDR] < 0.01; Supplementary Table  
160 4). These data pinpoint *pannier* as the major gene regulating the elytral colour pattern  
161 variation in *H. axyridis*.

162 We next investigated allele-specific polymorphisms at the *pannier* locus. We found  
163 that alleles of the first intronic region of *pannier* are more diverse than the surrounding  
164 genomic regions (Fig. 5a, the whitish regions in the middle), whereas the same allele in  
165 different strains shows conserved fragments distributed throughout the region (Fig. 5a, blue  
166 bars,  $h^C$ [F2-3]– $h^C$ [NT6]). In comparisons between the alleles, we consistently found traces of  
167 large inversions in the upstream half of the first intron (reddish lines in Fig 5a,  $h^C$ – $h^A$ ,  $h^A$ – $h$ ,

168  $h-h^C$ ; 56 kb–76 kb in size). However, we found only a single non-synonymous substitution in

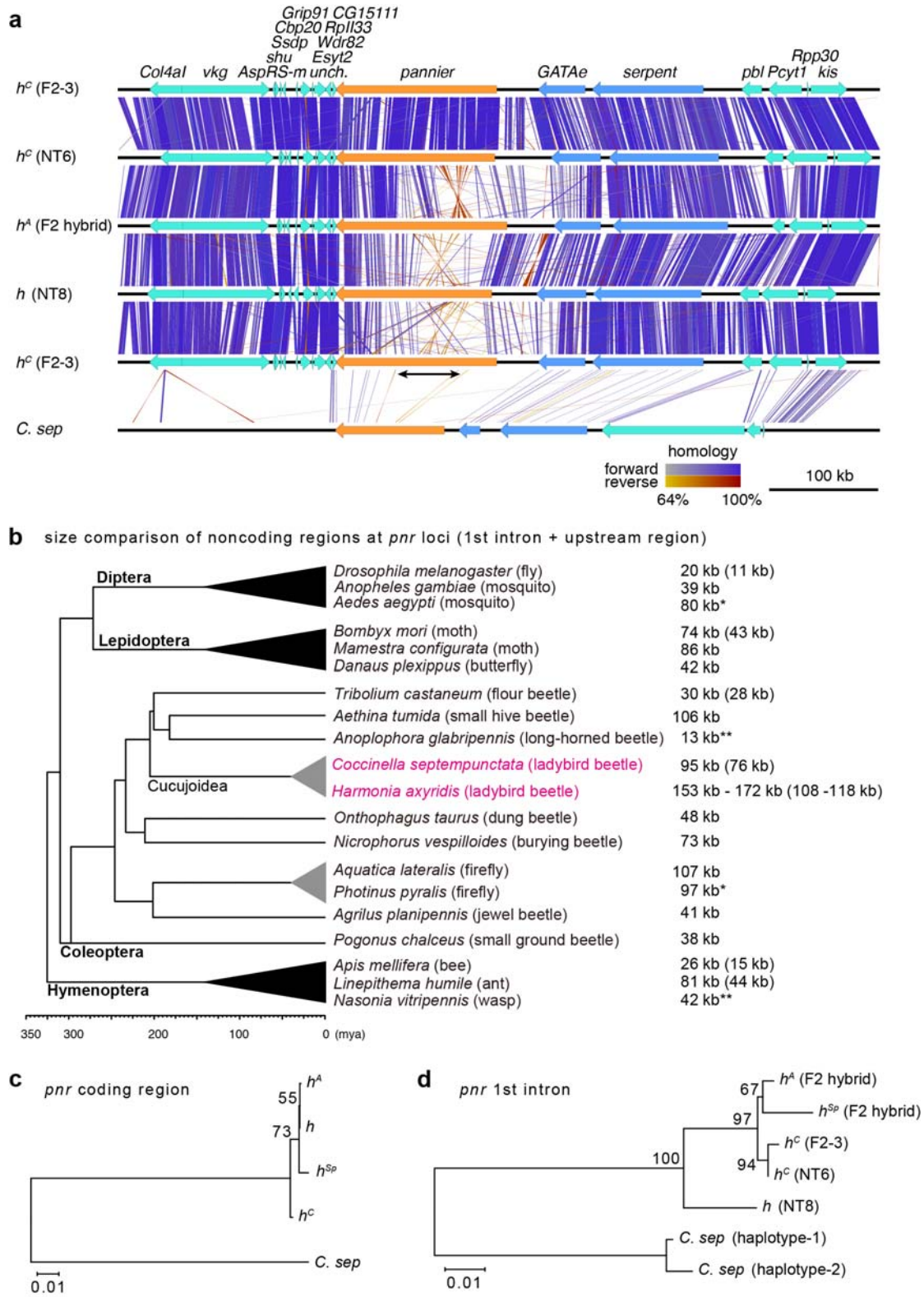
169 the region not conserved among organisms (G235V,  $h^{Sp}$ ), (Supplementary Figure 5, 6),

170 suggesting that cis-regulatory differences in the 1st intronic region of *pannier* are the major

171 cause of intraspecific colour variation.

172

173 **Figure 5. Intraspecific diversification and interspecific conservation of the 1st intronic**  
174 **region of *pannier* in ladybird beetles.** **a**, Sequence comparison of the genomic region  
175 surrounding the *pannier* locus. 700 kb genomic sequences surrounding the *pannier* locus  
176 were extracted from the genome assembly of each allele in *H. axyridis* ( $h^C$ ,  $h^A$ ,  $h$ ) and *C.*  
177 *septempunctata* (*C. sep*). Strain names are given in parentheses. Arrows indicate genes  
178 predicted by the exonerate programme (Orange, *pannier*; Blue, GATA transcription factor  
179 genes paralogous to *pannier*; Green, other genes). Gene names are listed at the top. Vertical  
180 or diagonal bars connecting adjacent genomic structures indicate BLAST<sup>47</sup> hit blocks (bluish,  
181 forward hit; reddish, reverse hit). The colour code for colouring the bars is at the bottom. The  
182 upper half (1st intron) of the *pannier* locus is diversified (whitish) between different  $h$  alleles  
183 in *H. axyridis*, and shows traces of inversions (crossed reddish bars). Several intronic  
184 sequences are conserved between *H. axyridis* and *C. septempunctata* (bars located in the  
185 upper half of *pannier* in *C. sep*). The black arrow indicates the region specifically expanded  
186 in *H. axyridis*. **b**, Overview of the size of the upper noncoding regions (the 1st intron + the  
187 upper intergenic region) at the *pannier* locus in holometabolous insects. The topology of the  
188 phylogenetic tree of surveyed insects is adapted from ref. <sup>48</sup> (Coleoptera), and TIMETREE<sup>49</sup>  
189 (Diptera, Hymenoptera, Lepidoptera). The sizes of the 1st intron are given in parenthesis if  
190 cDNA information was available. *H. axyridis* has the largest noncoding sequence at the  
191 *pannier* locus. In some species, synteny of the three paralogous GATA genes was broken up  
192 by translocation (\*) or insertion (\*\*). **c, d**, ML phylogenetic trees constructed with nucleotide  
193 sequences of *pannier* coding region (**c**), and those of the conserved region in the 1st intron (**d**).  
194 The trees were drawn to scale with branch lengths measured in the number of substitutions  
195 per site. Bootstrap values were calculated from 1000 resampling of the alignment data.



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198                    Moreover, we found that in *H. axyridis*, the size of upstream noncoding  
199 sequences of the *pannier* locus (including the 1st intron and the upstream intergenic region)  
200 are 46–65 kb larger than the currently available corresponding genomic sequences of the  
201 other holometabolous insects (Fig. 4b, *H. axyridis*, 153–172 kb; other holometabolous  
202 insects, 13–107 kb). Comparison of the exon-intron structures of *H. axyridis* to those of some  
203 of the holometabolous insects also suggested that especially the 1st intron of *pannier* is  
204 expanded in *H. axyridis* (*H. axyridis*, 108–118 kb; the other holometabolous insects, 11–44  
205 kb). The expanded region in *H. axyridis* included at least four transcription initiation sites of  
206 *pannier* transcripts (Fig. 4c, *pnr-1A–4B*). In addition, in this region, several known  
207 DNA-binding motifs of transcription factors involved in *Drosophila* wing formation were  
208 more enriched allele-specifically than those in the other genomic regions (Table 1a). For

209 example, the highly conserved Scalloped (SD) DNA binding motif of the insect wing selector

210 transcription factor complex Vestigial/Scalloped<sup>24, 25</sup> occurred frequently in upstream and

211 downstream regions of the first intron of *pannier* specifically in the  $h^C$  allele (Table 1a, the

212 upstream and downstream regions of the 1st intron, *sd*; Supplementary Table 5).

213

214

**Table 1 | Known DNA binding motifs enriched in the non-coding regions of the *pannier* loci.**

Enriched DNA binding motifs of *Drosophila* transcription factors involved in wing formation are listed (p < 0.05).

**a. Allele- or Specie-specifically enriched motifs**

strain	upstream intergenic region	upstream region of 1st intron	downstream region of 1st intron
<i>h<sup>C</sup></i>	<i>EcR, foxo</i>	<i>Myc, en, exd, HLH106, h, ss, pan Mad, Mnt, pad, Poxn, <u>sd</u>, tgo, tai, vvl</i>	<i>B-H1, ab, crc, crol, Dr, rn, <u>sd</u></i>
<i>h<sup>A</sup></i>	<i>exd, pan, vvl</i>	<i>ab, Spvs, brk, usp, pnr</i>	<i>usp</i>
<i>h</i>	<i>kni, rn, sqz</i>	<i>ato, ab, Ets21C, rn, sqz</i>	<i>al, B-H1, lms, nub, sqz, tup</i>
<i>C. sep</i>	<i>ato, EcR, h, ss, tgo</i>	<i>B-H2, eg, tai, pnr, tgo</i>	<i>E(spl)mβ, ken, brk, tgo, h, tai</i>

**b. Commonly enriched motifs**

group	upstream intergenic region	upstream region of 1st intron	downstream region of 1st intron
<i>Harmonia</i>	<i>Mad, brk, ab, taxi, tgo</i>	<i>lms, exd, hth, tgo, tai, Mad al, brk, tx, E(spl)mβ, tgo</i>	<i>Dr, en, inv, Med, sens, slou, unpg</i>
<i>Harmonia &amp; Coccinella</i>	<i>Mad</i>	<i>lms, exd, hth, tgo, tai, Mad</i>	—

**c. Region size (bp)**

strain	upstream intergenic region	upstream region of 1st intron	downstream region of 1st intron
<i>h<sup>C</sup></i>	38,192 (F2-3) / 51,566 (NT6)	71,107 (F2-3) / 76,264 (NT6)	47,450 (F2-3) / 44,236 (NT6)
<i>h<sup>A</sup></i>	42,826	75,417	52,210
<i>h</i>	40,778	67,340	67,340
<i>C. sep</i>	20,370	56,564	18,432

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216



217 Furthermore, the RNA-seq data for the  $h^C$  background also revealed that the *sd* co-activator  
218 gene *vestigial* was the only transcription factor gene that was significantly upregulated in the  
219 future black region from early pupal stages (Supplementary Figure 7), implicating Vestigial  
220 as one of the upstream trans-regulatory factors acting together with Sd to form the  
221 two-spotted elytral colour pattern of  $h^C$ . It is noteworthy that the non-coding region of  
222 *pannier* in each allele possesses putative DNA-binding motifs that can respond to variety of  
223 developmental contexts such as anterior-posterior patterning<sup>26-28</sup> (*en*, *inv*), wing fate  
224 specification<sup>17,24,29</sup> (*sd*), hinge-wing blade patterning<sup>17,18,30,31</sup> & wing vein patterning<sup>29, 32-34</sup>  
225 (*nub*, *rn*, *ab*, *al*, *B-H1*, *B-H2*, *ss*, *hth*, *exd*, *kni*, *brk*, *vvl*, *Mad*, *Med*, *h*), hormonal cues<sup>35, 36</sup> (*EcR*,  
226 *usp*, *tai*), and auto-regulation (*pnr*) (Table 1a). These results suggest that allele-specific

227 elytral colour patterns of *H. axyridis* may be formed by integrating appropriate combinations  
228 of developmental contexts of wing formation shared among insects.

229           We further tested whether the regulatory function of the red/black colour pattern in  
230 elytra is a conserved or a derived aspect of *pannier* function in ladybird beetles using the  
231 seven-spotted ladybird beetle, *Coccinella septempunctata*, which shows a monomorphic  
232 seven-spotted elytral colour pattern. The *pannier* mRNA was detected in the larval elytral  
233 primordium, was upregulated from 24h AP to 96h AP, and preferentially expressed in the  
234 black spots of elytra in *C. septempunctata* similar to that in *H. axyridis* (Supplementary  
235 Figure 8). The black-to-red switching phenotype was also observed in *C. septempunctata*  
236 adults treated with larval RNAi targeting *pannier* (Fig. 3a, *C. septempunctata*). These data  
237 suggest that the elytral colour-patterning function of *pannier* may be conserved at the

238 inter-genus level in ladybird beetles. To investigate the putative regulatory sequences at the  
239 *pannier* locus, we performed *de novo* assembly of the *C. septempunctata* genome using a  
240 linked-read sequencing platform (10x Genomics Chromium system), and obtained a  
241 contiguous genomic scaffold including the *pannier* locus (2.41 Mb, Supplementary Figure 4d,  
242 Supplementary Table 3). Whereas the noncoding sequences of *C. septempunctata pannier*  
243 are enriched with several species-specific DNA binding motifs (Table 1a, *C. sep*), we found  
244 DNA-binding motifs commonly enriched between *Harmonia* and *Coccinella*, which are  
245 associated with wing vein formation and wing/body wall patterning (*Mad*, *hth* and *exd*)<sup>29,31,32</sup>  
246 (Table 1b). Therefore, co-option of such wing developmental modules in the regulatory  
247 region may have facilitated acquisition of a novel expression domain of *pannier* in pupal  
248 elytral blades in ladybird beetles.

249           In order to explore the history of the emergence of elytral colour patterns in *H.*  
250 *axyridis*, we also performed a molecular phylogenetic analysis focusing on the highly  
251 conserved *pannier* intronic sequences shared among *H. axyridis* alleles and *C.*  
252 *septempunctata* (3 blocks, totalling 1.1 kb in length, Supplementary Data 1). The maximum  
253 likelihood (ML) phylogenetic tree inferred from nucleotide sequences of the *pannier* coding  
254 region did not resolve the phylogenetic relationship among the alleles in *H. axyridis* to a  
255 satisfactory level (Fig. 5d, bootstrap values < 75). However, the ML tree inferred from the  
256 conserved intronic sequence suggested that in *H. axyridis* the contrasting colour patterns of  
257 the *h* allele (black spots in red background) and the other three alleles (red spots in black  
258 background) diverged first. The latter three alleles diverged more recently (Fig. 5e, bootstrap  
259 values > 90).

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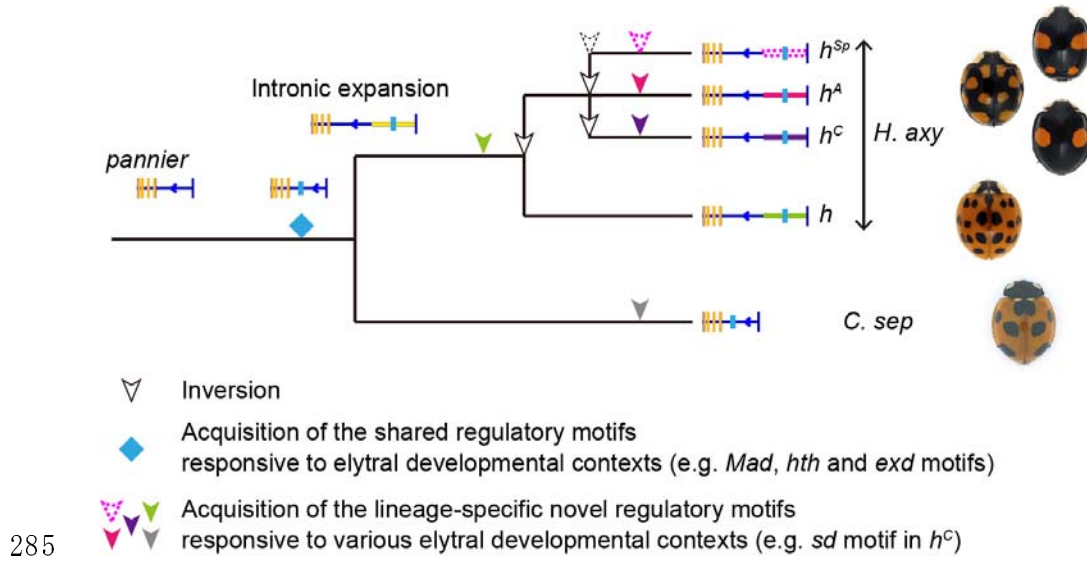
261 **Discussion**

262           The *pannier* locus identified in this study appears to be the key genetic locus  
263 responsible for the origin of large-scale intraspecific variation genetically linked to the *h*  
264 locus in ladybird beetles<sup>1,2</sup>. Based on the results presented in this study, we propose an  
265 evolutionary model that might underlie the high level of diversification of the intraspecific  
266 elytral colour patterns of *H. axyridis*. In addition, we also discuss the underlying evolutionary  
267 developmental backgrounds specific to ladybird beetles.

268           The common ancestor of *Harmonia* and *Coccinella* (Coccinellinae) diverged more  
269 than 33.9 million years ago, according to molecular phylogenetic analyses and fossil  
270 records<sup>37, 38</sup>. Therefore, the elytral colour-patterning function of *pannier* shared between *H.*  
271 *axyridis* and *C. septempunctata* was most likely acquired before this divergence event. The

272 1.1 kb sequence blocks in the 1st intron of *pannier* conserved between *H. axyridis* and *C.*  
273 *septempunctata* are a likely candidate for a regulatory element associated with the ladybird  
274 beetle-specific elytral expression of *pannier* in the pupal elytra. The effects of enhancer  
275 activities of these sequence blocks have not yet been experimentally addressed. However, the  
276 acquisition of such regulatory sequences during evolution would have coincided with the  
277 acquisition of the elytral colour patterning function of *pannier* (Fig. 6, blue diamond). These  
278 conserved sequence blocks are located in the expanded intronic region specific to *H. axyridis*  
279 (Fig. 5a, black arrow). Therefore, the expansion of the 1st intron in the ancestral lineage of *H.*  
280 *axyridis* (Fig. 6, intronic expansion) might be one of the events that facilitated diversification  
281 of the intraspecific elytral colour patterns.  
282

283 Figure 6. Evolutionary model for the acquisition of the highly diversified intraspecific elytral  
284 colour patterns of *H. axyridis*. See details in the Discussion.



286

287           In the genus *Harmonia*, colour patterns similar to those encoded by the *h* allele and  
288 those of *C. septempunctata* (black spots in red background) are commonly observed. Also  
289 the position of the spots is similar across species (e.g. *H. quadripunctata*, *H. octomaculata*,  
290 and *H. dimidiata*). Therefore, we speculate that the intronic sequence of *pannier* in the *h*  
291 allele of *H. axyridis* might retain a repertoire of regulatory sequences acquired in a common  
292 ancestor of the genus *Harmonia* (Fig 6, green arrowhead). However, in the ancestral lineage  
293 of *H. axyridis*, the regulatory region of *pannier* appears to have been modified to generate  
294 novel colour patterns of the recently diverged alleles ( $h^C$ ,  $h^{Sp}$  and  $h^A$ ; red spots in black  
295 background; Fig. 6 magenta, red and purple arrowheads). The 46–65 kb-scale intronic region  
296 at the *pannier* locus that is specifically expanded in *H. axyridis* (Fig. 6, intronic expansion,  
297 yellow box) might have facilitated accommodation of the allele-specific regulatory motifs



298 responsible for the diversified colour pattern of elytra. In addition, traces of inversions in this  
299 region consistently found in allele comparisons suggest that repeated inversions in this region  
300 (Fig. 6, white arrowheads) created opportunities to diverge the noncoding sequence of  
301 *pannier* to successively generate novel diverse alleles within a species by suppressing  
302 recombination within this region. Such inversion events would have occurred in the common  
303 ancestor of *H. axyridis* and its reproductively isolated sister species, *H. yedoensis* because the  
304 major elytral colour patterns are shared between the two species<sup>39</sup>. Large-scale chromosomal  
305 inversion is believed to be one of the major driving forces generating and maintaining  
306 intraspecific morphological variation within a species<sup>40–43</sup>. Our study exemplifies that not  
307 only a single inversion event but also repeated inversion events at an expanded intron can  
308 lead to the acquisition of novel morphological traits within a species.

309                   From the viewpoint of evolutionary developmental biology it is noteworthy that in

310    *H. axyridis*, of all of the developmental genes known to regulate colour pattern and

311    pigmentation, a single gene, *pannier*, is responsible for the major classes of intraspecific

312    entire wing colour pattern diversification. This evolutionary pattern contrasts with that of the

313    intensely studied warning signals of *Heliconius* butterflies. In this case, more than 10 loci

314    regulate multiple intraspecific wing colour patterns prevailing in the population<sup>44</sup>. This

315    difference in evolutionary mechanisms may stem from a paucity of available options of

316    evolvable genes in the gene regulatory network of elytral colour patterning. Ladybird beetles

317    diverged from ancestral species of Cucujoidea<sup>37</sup> (Fig. 5a, Cucujoidea), leaf-litter or

318    rotten-tree dwelling insects. Thus, the ancestor of ladybird beetles would have had far less

319    colourful and more simply patterned forewings (elytra) than the ancestors of butterflies,

320 moths. Therefore, these ancestors presumably would have possessed far fewer colour pattern  
321 regulatory genes. In *H. axyridis*, this developmental constraint may have led to the selection  
322 of *pannier* as the major evolvable gene to a signal-integrating ‘input-output’ regulatory  
323 gene<sup>45,46</sup>. This might have generated more than 200 colour patterns genetically tightly linked  
324 to the *h* locus by utilising the expanded regulatory DNA sequence. Future research aiming to  
325 identify specific regulatory inputs to *pannier* will help clarify the regulatory mechanisms  
326 underlying the generation of highly diverse intraspecific polymorphism at the interspecific  
327 level. Another important issue to clarify whether *pannier* is indeed the hotspot of  
328 morphological evolution in ladybird beetles is whether *pannier* is responsible for the  
329 remaining >20 minor colour patterns in *H. axyridis*.

330 **Methods**

331 **Insects**

332           Laboratory stocks of *H. axyridis* and *C. septempunctata* were derived from field  
333 collections in Japan. They were reared at 25°C and usually fed on artificial diet<sup>50</sup>, or fed on  
334 the pea aphid *Acyrtosiphon pisum* (kindly provided by Dr. T. Miura) for egg collection.

335 Larvae and pupae analysed in this study were not sexed.

336

337 **Phenoloxidase (PO) activity staining**

338           Pupa elytral discs were dissected in a potassium phosphate buffer (100 mM  
339  $\text{KH}_2\text{PO}_4/\text{K}_2\text{PO}_4$ , 150 mM NaCl, pH 6.3) on ice. PO staining was performed using 0.4 mg/ml  
340 dopamine as a substrate for 2 hours at room temperature as previously described<sup>13</sup>. After

341 washing several times in the potassium phosphate buffer containing 0.3% Triton-X100 and  
342 mounted in this solution. Images were captured with a stereoscopic microscope (MZ FLIII,  
343 Leica) equipped with a digital camera (DP70, Olympus).

344

#### 345 **Histological analysis**

346 To visualise tissue morphology and PO active tissues, pharate adult elytra dissected  
347 in ice-cold PBS (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) at 96 h AP or  
348 those after PO activity staining were fixed with 4% paraformaldehyde (PFA) in PBS for 15  
349 min on ice and for 75 min at room temperature. After fixation, the elytra were washed several  
350 times in 100% methanol and stored in 100% methanol at -20°C until use. After dehydration,  
351 the elytra were embedded in 4% carboxymethyl cellulose (CMC; FINETEC), and were

352 frozen in hexane cooled with dry ice. The freeze-embedded elytra were stored at  $-80^{\circ}\text{C}$  until  
353 use. The  $6\ \mu\text{m}$  frozen sections were prepared as described previously<sup>51</sup> using an adhesive film  
354 (Cryofilm Type 1; FINETEC). Sections of the PO activity stained elytra were mounted in  
355 PBS and photographed using IX70. For nuclear and F-actin staining, sections were treated  
356 with  $2.5\ \mu\text{g}/\text{ml}$  propidium iodide,  $1\ \text{mg}/\text{ml}$  RNase A and  $5\ \text{U}/\text{ml}$  AlexaFluor 488 phalloidin  
357 (Molecular Probes) for 1 hour at  $37^{\circ}\text{C}$  under a dark condition. After washing three times in  
358 PBS, the sections were mounted in an antifade reagent (FluoroGuard<sup>TM</sup>; Bio-Rad), and  
359 images were captured with a confocal laser-scanning microscope (LSM 510; Carl Zeiss).

360 For localisation of carotenoid, elytra at 96 h AP were embedded and sectioned as  
361 described above. All procedures were rapidly performed to prevent diffusion of carotenoids.  
362 The sections were dried for 1 min, mounted in PBS and immediately photographed using

363 IX70.

364

365 **cDNA cloning**

366 Larval and pupal elytral discs and pharate adult elytra of *H. axyridis* ( $h^C$ ) and *C.*

367 *septempunctata* were dissected in PBS on ice. Soon after dissection, the tissues were frozen

368 in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. Total RNA was extracted from each sample

369 using TRIzol Reagent (Invitrogen) or RNeasy Micro Kit (Qiagen) according to the

370 manufacture's instructions, and treated with 2 U DNase I (Ambion) for 30 min at  $37^{\circ}\text{C}$ . The

371 first-strand cDNA was synthesised with SMARTer PCR cDNA Amplification Kit (Clontech)

372 using 1  $\mu\text{g}$  of total RNA according to the manufacture's instructions. *H. axyridis* and *C.*

373 *septempunctata* cDNA fragments were amplified by reverse transcription-polymerase chain

374 reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) with the primers listed in  
375 Supplementary Table 6–8. The PCR product was cloned into the *EcoR* V site of the  
376 pBluescript KS+ vector (Stratagene) or pCR4-TOPO vector (TOPO TA Cloning Kit;  
377 Invitrogen). The nucleotide sequences of the PCR products were determined using a DNA  
378 sequencer 3130 genetic analyser (Applied Biosystems). The SNPs in open reading frame  
379 (ORF) of *pannier* were determined through direct sequencing of the PCR products treated  
380 with ExoSAP-IT (Affymetrix). Sequencing was performed by DNA sequencing service  
381 (FASMAC) using the primers listed in Supplementary Table 6. Sequence analysis was  
382 carried out using DNASIS (Hitachi Software Engineering) or ApE<sup>52</sup> (version 2.0.45)  
383 software. Nucleotide sequences and deduced amino acid sequences were aligned with



384 ClustalW in MEGA<sup>53</sup> software (version 7.0.18) and the alignment figure was generated using

385 Boxshade<sup>54</sup> (version 3.21).

386

### 387 **Gene expression analysis by RT-PCR**

388 For the gene expression analysis in each developmental stage, elytral tissues of

389 three individuals of *H. axyridis* ( $h^C$ ) and *C. septempunctata* were dissected as described

390 above. Six elytral tissues from each sampling stage were pooled in one tube. Total RNA

391 extractions and the subsequent first-strand cDNA syntheses (using 425 ng and 267 ng of total

392 RNA for *H. axyridis* and *C. septempunctata* samples, respectively) were performed as

393 described above. Three  $\mu$ l of 100 and 62.8 times diluted *H. axyridis* and *C. septempunctata*

394 first-strand cDNA was used as a template for PCR, respectively. The PCR cycle numbers are

395 35 for all genes. A set of primers #1 and #2 for each gene was used for this analysis

396 (Supplementary Table 7).

397 For the gene expression analysis in the future red and black regions, red and black

398 regions of pharate adult elytra at 84 h AP were bored with a needle. Internal diameters of 0.7

399 and 0.6 mm were used for *H. axyridis* and *C. septempunctata*, respectively. In the case of *C.*

400 *septempunctata*, elytra stained with PO activity were used for boring since carotenoid

401 localisation was not observable unlike *H. axyridis*. cDNA synthesis was performed as

402 described above, using as much total RNA as we could extract. 20 µl of 10 times diluted

403 first-strand cDNA was used as a template for PCR. The PCR cycle numbers are 45 cycles for

404 *Ha-pnr*, 38 cycles for *Ha-rp49*, 47 cycles for *Cs-pnr* and 40 cycles for *Cs-rp49*. A set of

405 primers #3 and #4 for each gene was used for this analysis other than *rp49*. *Ha-rp49* and

406 *Cs-rp49* were used as internal controls. Reactions without reverse transcriptase were  
407 performed with cDNA synthesis as negative control samples for the RT-PCR experiments.  
408 No band was detected in these reactions for all genes. The primers used for this analysis are  
409 described in Supplementary Table 7.

410

#### 411 **Larval RNAi**

412 dsRNA synthesis and microinjection into larvae were performed as previously  
413 described<sup>19</sup>. The cloned cDNA fragments amplified by PCR were used as templates for  
414 dsRNA synthesis. Approximately 1.4 to 2.7 µg and 1.4 to 2.0 µg of the dsRNAs of *Ha-pnr*  
415 and *Cs-pnr* were injected into two-day-old fourth (final) instar larvae, respectively.  
416 Approximately 2.0 to 2.7 µg and 1.4 to 2.7 µg of the *EGFP* dsRNA were injected into *H.*

417 *axyridis* and *C. septempunctata* larvae as negative controls, respectively. Different amount of

418 dsRNA for each gene in this range gave no difference in phenotypic effects (data not shown).

419 In order to give enough time for the completion of pigmentation, images of adults were

420 captured at more than 2 days after eclosion using a digital microscope (VHX-900, Keyence).

421

#### 422 ***In situ* hybridisation**

423           Essentially the same protocol for whole mount pupal antennal primordia of the silk

424 moth<sup>55</sup> was used. To increase RNA probe penetrance in elytral epidermis covered with

425 sclerotised cuticle at 76–84 h AP, the peripheral edge of an elytron was cut off, and then,

426 ventral and dorsal elytral epidermis layers appressed together were carefully separated with

427 fine forceps after fixation. To reduce non-specific probe hybridisation, fixed, separated and

428 detergent-permeabilised elytra epidermal samples were stored in 100% methanol for more  
429 than 12 hours at  $-30^{\circ}\text{C}$ , and prehybridisation treatment was extended to two overnights. The  
430 ventral epidermis samples were not used for analysis because of high non-specific  
431 background signals. *pannier* antisense probes were designed at 5' and 3' regions of ORF  
432 excluding the two conserved GATA zinc finger coding regions in the middle to prevent  
433 cross-hybridisation with other GATA family genes. The PCR primers used to amplify the  
434 template DNA for *in vitro* RNA probe synthesis were listed in Supplementary Table 9. Briefly,  
435 *pannier* ORF fragment was amplified by RT-PCR using cDNA from 72 h AP ( $h^C$ ), and cloned  
436 into pCR4-TOPO vector (Invitrogen). Sense and antisense probe templates were amplified  
437 from the cloned cDNA. Sense and antisense riboprobes were transcribed using the flanking  
438 T7, T3 or SP6 promoter sequences. Mixture of 5' and 3' probes was used for hybridisation.

439

440 ***De novo* genome assembly of *H. axyridis***

441           A single female adult from F2-3 strain sibcrossed for 3 generations ( $h^C$ ) was used  
442 for the first version of *de novo* genome assembly of *H. axyridis*. Genomic DNA was extracted  
443 using DNeasy Blood and Tissue Kit (Qiagen). Paired end (300 bp and 500 bp) and mate pair  
444 (3 kb, 5 kb, 8 kb, 10 kb, 12 kb and 15 kb) libraries were constructed using TruSeq DNA  
445 PCR-Free LT Sample Prep Kit and Nextera Mate Pair Sample Prep Kit (Illumina) following  
446 the manufacturer's protocols. Sequencing libraries were run on Illumina HiSeq2500  
447 sequencers. In total, we generated 133.6 Gb of raw sequence data for *de novo* genome  
448 assembly. Genome assembly was performed using the Platanus v1.2.1.1 assembler<sup>56</sup> after  
449 removal of adapter sequences and error correction (SOAPec v2.01)<sup>57</sup>.

450

451 **Re-assembly of the genomic sequence at the *pannier*-locus in the *H. axyridis* F2-3**

452 **sample**

453 Adaptor sequences and low-quality regions in paired-end and mate-pair reads were

454 trimmed using `Platanus_trim`<sup>58</sup> (version 1.0.7) with default parameters. Trimmed reads were

455 assembled by `Platanus2`<sup>59</sup> (version 2.0.0), which was derived from `Platanus`<sup>56</sup> to assemble

456 haplotype sequences (i.e. haplotype phasing) instead of consensus sequences. Procedures of

457 `Platanus2` are briefly described as follows:

458 (1) De Bruijn graphs and scaffold graphs are constructed without removal of bubble

459 structures caused from heterozygosity. Paths that do not contain junctions correspond to

460 assembly results (scaffolds). Scaffold pairs in bubbles represent heterozygous haplotypes.

461 (2) Paired-ends or mate-pairs are mapped to the graphs to detect links between bubbles, and  
462 linked bubbles are fused to extend haplotype sequences.

463 (3) Each haplotype (contig or scaffold) is independently extended by modules of *de novo*  
464 assembly derived from Platanus.

465 (4) Homologous pairs of haplotype scaffolds are detected using bubble information in the  
466 initial de Bruijn graph.

467 (5) Steps 1–4 are iterated using various libraries (paired-ends or mate-pairs).

468 (6) Homologous pairs of scaffolds are formatted into bubble structures as output. For each  
469 pair, longer and shorter scaffold were called "primary-bubble" and "secondary-bubble",  
470 respectively. Primary-bubbles, secondary-bubbles and non-bubble scaffolds are collectively  
471 called "phased-scaffolds".



472 In addition, Platanus2 can connect primary-bubbles and non-bubble scaffolds to construct  
473 long "consensus scaffolds", which consists of mosaic structure of haplotypes (i.e. paternal  
474 and maternal haplotypes are mixed). Employing the strategy of Platanus2, certain highly  
475 heterozygous regions were expected to be assembled contiguously compared to Platanus.  
476 Using the markers of the responsible region of wing pattern (*pannier*-locus), we found that  
477 two long bubbles and one short non-bubble scaffold corresponded to the locus. Consequently,  
478 one consensus scaffold was constructed from these phased scaffolds, which covering the  
479 breakpoint markers at the *pannier* locus. We used that consensus scaffold (3.13 Mb) for the  
480 downstream *in silico* sequence analyses. We assessed the completeness of the genome  
481 assemblies using BUSCO<sup>60</sup> (version 3.0.2, Insecta dataset [1,658 orthologs]).

482

483 **DNA sequencing for *de novo* genome assembly by long read and linked-read platforms**

484 High molecular weight (HMW) genomic DNA was extracted using QIAGEN

485 Genomic-tip 100/G (QIAGEN) according to the manufacturer's instructions. The

486 concentrations and qualities of the extracted HMW genomic DNA were evaluated using

487 Qubit dsDNA, and RNA HS kits (Thermo Fisher).

488 For library preparation for 10x Genomics Chromium system, one pupa ( $h^C$  [NT6

489 strain] and  $h$  [NT8 strain]) or one adult ( $h^A$  [F2 adult progenies in genetic cross  $h^A \times h^{Sp}$ ] and

490 *C. septempunctata* [MD-4 strain]) was used. Size selection by BluePippin (range:

491 50kb–80kb, Sage Science) was performed only for  $h^A$  genomic DNA used in 10x linked-read

492 library preparation.

493                   Preparation of Gel Bead-in-Emulsions (GEMs) for each 10x Genomics Chromium

494   library was performed using 0.5–0.6 ng of HMW genomic DNA according to the

495   manufacturer’s instructions. The prepared GEMs were quality checked using Qubit dsDNA

496   HS kit (Thermo Fisher) and Bioanalyzer (Agilent), and processed with Chromium Controller

497   (10x Genomics). The constructed DNA libraries were quality checked again in the same way.

498   Sequencing of the libraries was performed in the Hiseq X ten (Illumina) platform (1

499   library/lane) at Macrogen. In total, we generated 66.9 Gb, 64.6Gb, 64.9 Gb and 60.3 Gb of

500   raw reads for linked-read sequencing ( $h^C$ ,  $h^A$ ,  $h$ , and *C. septempunctata*, respectively).

501                   For library preparation for PacBio system, ten to eleven pupae ( $h^C$  [NT6 strain] and

502   *h* [NT8 strain]) or adults ( $h^A$  [F2 adult progenies in genetic cross  $h^A \times h^{Sp}$ ]) were used.

503 The libraries were prepared according to the 20-kb Template Preparation Using  
504 BluePippin™ Size-Selection System (Sage Science, MA, USA). In total, 4.31 Gb, 4.92 Gb  
505 and 4.44 Gb of insert sequences (approximately  $10 \times$  coverage of the genome, assuming a  
506 genome size of 423 Mb) were obtained from 4 to 5 SMRT cells for  $h^A$ ,  $h^C$  and  $h$ , respectively.

507

#### 508 ***De novo* assembly of 10x linked-reads**

509 For 10x linked-reads libraries of four samples (three *H. axyridis* and one *C.*  
510 *septempunctata*), Supernova (version 2.0.0)<sup>61</sup> was executed with default parameters except  
511 for the maximum number of used reads (the --maxreads option) to obtain the optimum  
512 coverage depth for Supernova (56 $\times$ ). For each sample, the value for --maxreads was  
513 determined as follows:

514 (1) Barcode sequences in raw linked-reads were excluded using "longranger basic" command

515 of Long Ranger<sup>62</sup> (version 2.1.2), resulting in "barcoded.fastq" file.

516 (2) Adaptor sequences and low-quality regions in "barcoded.fastq" were trimmed using

517 Platanus\_trim (version 1.0.7) with default parameters.

518 (3) 32-mers in the trimmed reads were counted by Jellyfish<sup>63</sup> (version 2.2.3) using the

519 following two commands and options:

520 \$ jellyfish count -m 32 -s 20M -C -o out.jf barcoded\_1.trimmed barcoded\_2.trimmed

521 \$ jellyfish histo -h 1000000000 -o out.histo out.jf

522 In summary, all 32-mers in both strands (-C) were counted and distribution of the number of

523 occurrences without upper limit of occurrences (-h 1000000000).

524 (4) The haploid genome size was estimated using the custom Perl script. For the distribution  
525 of the number of 32-mer occurrences ("out.histo"), the number of occurrences corresponding  
526 to a homozygous peak was detected, and the total number of 32-mers was divided by the  
527 homozygous-peak-occurrences. Here, 32-mers whose occurrences were small (< the number  
528 of occurrences corresponding to the bottom between zero and heterozygous peak) were  
529 excluded for the calculation to avoid the effect from sequencing errors.

530 (5) The values for --maxreads were calculated as follow:

531  $\text{estimated-haploid-genome-size} / \text{mean-read-length-of-barcoded.fastq} \times 56$

532 As a result, we obtained the scaffolds including the genes surrounding *H.*

533 *axyridis-pannier* ( $h^C$  [NT6], 2.74 Mb;  $h^A$  [F2 hybrid], 1.42 + 1.61 Mb;  $h^C$  [NT8] 2.79 Mb),

534 and homologous regions in *C. septempunctata* (haplotype 1, 10.16 + 2.41 Mb; haplotype 2,

535 10.13 + 2.44 Mb). We used those sequences for the downstream *in silico* analyses. We  
536 assessed the completeness of the genome assembly using BUSCO<sup>62</sup> (version 3.0.2, Insecta  
537 dataset [1,658 orthologs]).

538

#### 539 **Gap filling of the genomic scaffolds at the *pannier* locus**

540           Concerning the genome assemblies of *H. axyridis*, we used minimap2<sup>64</sup> (ver. 2.9)  
541 and PBjelly<sup>65</sup> (ver. PBSuite\_15.8.24) software to fill gaps around the *pannier* locus. In each  
542 genome of three strains of *H. axyridis*, we first mapped PacBio reads to the assembly  
543 generated from the 10x linked-reads using minimap2. Then, we chose PacBio reads mapped  
544 to the scaffold containing *pannier* gene. These PacBio reads were subjected to gap-filling of

545 the scaffold with PBjelly. We obtained gap-free nucleotide sequences spanning the entire

546 *pannier* locus and the upstream intergenic regions.

547           Concerning the genome assembly of *C. septempunctata*, there was a single gap

548 estimated to be 15kb long by Supernova programme in the 1st intron of *pannier* locus. We

549 handled this gap region as repeated N, and included it in the downstream *in silico* analyses.

550

551 **Validation of the *pannier*-locus scaffold of Platanus2 for the *H. axyridis* F2-3 sample**

552 For the *H. axyridis* F2-3 sample, trimmed reads of the 15 kbp-mate-pair library were mapped

553 to the consensus scaffold set of Platanus2 using BWA-MEM<sup>66</sup> (version 0.7.12-r1039) with

554 default parameters. Next, a consensus scaffold corresponding to *pannier*-locus was



555 segmented into 2 kbp-windows, and links between windows (the number of mate-pairs  $\geq 3$ )

556 were visualised by Circos<sup>67</sup> (version 0.69-6).

557

558 **Additional comment for the editor and reviewers.**

559 **Although Platanus2 is not open currently, we plan to release the same version (2.0.0) of**

560 **Platanus2 at the web site of Platanus (<http://platanus.bio.titech.ac.jp/>) by the publication of**

561 **this study.**

562

563 **Resequencing of *Harmonia* genome for choosing appropriate strains for Genome Wide**

564 **Association Study (GWAS)**

565                    Genomic DNA was extracted from each of  $h^C$  (F6 strain),  $h^A$  (NT3 strain) and  $h^{Sp}$   
566                    (CB-5 strain), and used to create Illumina libraries using TruSeq Nano DNA Sample  
567                    Preparation Kit (Illumina) with insert size of approximately 400 bp. These libraries were  
568                    sequenced on the Illumina HiSeq 1500 using a 2 x 106-nt paired-end sequencing protocol,  
569                    yielding 84.7M paired-end reads. SNP site identification was conducted basically according  
570                    to GATK Best Practice<sup>68</sup> (ver. August 7 2015). After trimming adaptor sequences with  
571                    Cutadapt software (ver. 1.9.1)<sup>69</sup>, the sequence data were mapped to the *de novo* genome  
572                    assembly data using bwa software (ver.0.7.15, BWA-MEM algorithm)<sup>66</sup>. Sequences and  
573                    alignments with low quality were filtered using Picard<sup>70</sup> tools (ver. 2.7.1) and GATK<sup>68</sup>  
574                    software (ver. 3.6 and 3.7), and 73,4443 SNP markers in the strains were identified. The most

575 distantly related strains ( $h^C$  [F6 strain] and  $h^A$  [NT3 strain]) were selected by performing

576 phylogenetic analysis using SNPhylo<sup>71</sup> (Version: 20140701).

577

578 **Comparison of the scaffolds at the *pannier* locus among ladybird beetles**

579 For each pair of scaffolds, we constructed dot plots by performing

580 pairwise-alignment using "nucmer" programme in the MUMmer package<sup>72</sup> (version 3.1).

581 The options of nucmer were as follows: (1) *H. axyridis* vs. *H. axyridis*, Default parameters;

582 (2) *H. axyridis* vs. *C. septempunctata*; '-l 8 -c 20'. Alignment results (delta file) were input

583 into "mummerplot" programme to generate dot plots. Note that resultant gnuplot scripts

584 resulted from mummerplot were edited for visualization.

585           We also visualised the homology and structural differences between the 700  
586 kb-genomic region including *pannier* using Easyfig<sup>73</sup> (ver. 2s2.2). Short BLAST<sup>47</sup> hit  
587 fragments less than 500 bp, and putative short repeat sequences less than 1250 bp, which  
588 showed more than two BLAST hit blocks within the 700 kb region, were filtered using a  
589 custom Perl script. Exon-intron structures of putative genes in the 700 kb regions were  
590 obtained using Exonerate<sup>74</sup> (ver. 2.2.0) with the options '-m est2genome --showvulgar yes  
591 --ryo ">%qi length=%ql alnlen=%qal\n>%ti length=%tl alnlen=%tal\n" --showtargetgff yes  
592 --showalignment no --score 2000'. cDNA sequences cloned by RT-PCR or predicted by  
593 RNA-seq were used as queries. If a single cDNA unit was split into multiple fragments, we  
594 merged the fragments by performing exonerate search again using the cDNA sequences  
595 whose subsequences were substituted by the genomic hit fragments in the first exonerate

596 search as a query. Exonerate output files were converted to the GFF3 format using our  
597 bug-fixed version of the `process_exonerate_gff3.pl`<sup>75</sup> with the option '-t EST'. The GFF3 file  
598 and a FASTA format file of each scaffold were converted to a GENBANK format file using  
599 EMBOSS Seqret<sup>76</sup> programme (ver. 6.6.0.0) with the options '-fformat gff -osformat genbank  
600 '. The GENBANK format files corresponding to the 700 kb genomic sequences surrounding  
601 *pannier*, which were used as input files of Easyfig, were extracted using the  
602 `Genbank_slice.py`<sup>77</sup> Python script (ver. 1.1.0).

603

#### 604 **Flexible ddRAD-seq**

605 We newly constructed a flexible ddRAD-seq library preparation protocol to  
606 facilitate high-throughput ddRAD-seq analyses at low cost. We designed all enzymatic

607 reactions to be completed sequentially without DNA purification in each step to make the  
608 procedures simple. In addition, we designed 96 sets of indexed and forked sequencing  
609 adaptors compatible with Illumina platform sequencers (Supplementary Table 10).

610           Briefly, 100 ng of genomic DNA was first double-digested with 15 U of EcoRI-HF  
611 and 15 U of HindIII-HF in 20  $\mu$ l of NEB CutSmart Buffer (New England Biolabs) at 37°C for  
612 2 hours. 15  $\mu$ l of the digested DNA, 4 pmole of adaptor DNA, 10  $\mu$ mole of ATP, 400 Units of  
613 T4 DNA ligase were mixed in 20  $\mu$ l, incubated at 22 °C for 2 hours, and denatured at 65°C for  
614 10 minutes. Ligated library DNA fragments were purified with Agencourt AMPureXP  
615 (Beckman Coulter) according to the manufacturer's instructions. Library DNA fragments  
616 ranging form 300 bp to 500 bp were size-selected with Pippin Prep (Sage Science).  
617 Concentration of each library DNA was quantified using KAPA Library Quantification Kits

618 (Roche) according to the manufacturer's instructions. Sequence data were obtained by

619 applying 96 DNA libraries to a single lane of Hiseq 1500 (Illumina).

620

### 621 **Linkage map construction and GWAS**

622 A single  $h^C$  male (F6 strain) and a single virgin  $h^A$  female (NT3 strain) were crossed, and the

623 obtained F1 progenies were backcrossed with the F0 male ( $h^C$ , F6 strain). Finally, 183 adult

624 F2 progenies ( $h^C = 80$ ,  $h^C/h^A = 103$ ) and 2 F0 adults were collected for RAD-sequence

625 analysis, and stored at  $-30^\circ\text{C}$  until use. Genomic DNAs were extracted individually using an

626 automatic nucleic acid extractor (PI-50 $\alpha$ , KURABO). Briefly, each frozen ladybird beetle

627 and a zirconia bead were transferred to 2 ml plastic tube (Eppendorf) on ice. Immediately,

628 250  $\mu\text{l}$  of cold lysis buffer including Proteinase K and RNase, but not SDS was added to the

629 sample, and the tubes were vigorously shaken with a tissue grinder (Tissue Lyser LT, Qiagen)  
630 at 3000 rpm for 1 min. Then, 250  $\mu$ l of lysis buffer including SDS was added to each crushed  
631 sample, and processed with the programme for DNA extraction from mouse tail, according to  
632 the manufacturer's instructions. The extracted genomic DNA was diluted in 30  $\mu$ l of TE  
633 buffer. The DNA concentration of each sample was quantified using Qubit dsDNA BR Kit  
634 according to the manufacture's instructions (Thermo Fisher Scientific). We performed  
635 flexible ddRAD-seq using these genomic DNA samples. 0.6–6.0 millions (mean = 2.0  
636 millions) of 106 bp paired-end reads per sample were generated using two lanes of Hiseq  
637 1500 (Illumina) following the methods in the User Guide.

638 Mapping and polymorphic site calling were conducted as described in the  
639 resequencing analysis above except that the procedure for filtering duplicated reads using



640 Picard was eliminated because we did not amplified DNA library by PCR. Count data at each

641 SNP sites were extracted from the obtained vcf file using vcf\_to\_rqtl.py script in rtd

642 software<sup>78</sup> with the options '5.0 80'. To avoid programme errors, we modified the script to

643 skip the read depth data (DP) including characters in the GATK vcf file.

644 We constructed a linkage map using R/qtl<sup>79</sup> (version 1.42.8) and R/ASMap<sup>80</sup>

645 (version 1.0.2) R<sup>81</sup> packages according to the QTL mapping workflow for BC1 population of

646 *Jaltomata*<sup>81</sup>. Using the obtained csv file as an input, we eliminated the polymorph sites that

647 behaved as located on the X chromosome. In addition, individuals with low mapping quality,

648 and marker sites with low quality or highly distorted segregation patterns were eliminated as

649 well. Finally, 4419 markers sites, and 177 F2 individuals were used. The linkage map was

650 initially constructed with mstmap programme (R/ASMap) with the options 'dist.fun =

651 "kosambi", p.value = 1e-25', and highly linked linkage groups were merged manually. The  
652 markers consistently incongruent with neighboring markers were eliminated using  
653 correctGenotypes.py<sup>82</sup> script with the options '-i csvr -q 0.1 -t 4.0'.

654 Genome wide association study was conducted using calc.genoprob programme  
655 (R/qtl) with the options 'step=1, error.prob=0.001' and scanone programme (R/qtl) with the  
656 option 'model="binary"'. The result data were visualised with plot programme in R/ASMap.

657

#### 658 **Genetic association study focusing on the *pannier* locus**

659 In addition to the genetic cross in GWAS ( $h^C \times h^A$ ), two independent crosses ( $[h \times$   
660  $h^C]$  and  $[h^A \times h^{Sp}]$ ) were performed.

661                    In the former cross, a single  $h$  male (D-5 strain) and a single virgin  $h^C$  female  
662                    (F2-3-B strain) were crossed, and the obtained F1 progenies were sibcrossed. Finally, 80 F2  
663                    adult progenies ( $h^C = 30$ ,  $h^C/h = 34$ , and  $h = 16$ ) were collected for genotyping, and stored at  
664                     $-30^\circ\text{C}$  until use. In the latter cross, a single  $h^{Sp}$  male (CB-5 strain) and a single virgin  $h^A$   
665                    female (NT3 strain) were crossed, and the obtained F1 progenies were sibcrossed. Finally,  
666                    273 F2 adult progenies ( $h^{Sp} = 103$ ,  $h^{Sp}/h^A = 80$ , and  $h^A = 90$ ) were collected for genotyping,  
667                    and stored at  $-30^\circ\text{C}$  until use.

668                    Genomic DNA was extracted individually using the automatic nucleic acid  
669                    extractor (PI-50 $\alpha$ , KURABO) as described in the previous section, and diluted to  
670                    approximately 100 ng/ $\mu\text{l}$ . We searched for genotyping markers by amplifying and  
671                    sequencing the intronic region of the genes surrounding *pannier* with PCR. The individual

672 PCR was performed using approximately 100 ng of genomic DNA and Q5 DNA polymerase  
673 (New England Biolabs) with 45 cycles. The primers used, the markers identified and the  
674 typing results are summarised in Supplementary Table 2.

675

#### 676 **RNA-seq analysis**

677           The total RNA extraction procedure for RNA-seq is essentially the same as that for  
678 the gene expression analysis in the presumptive red and black regions by RT-PCR. The same  
679 strain used for *de novo* genome sequencing (F2-5 strain,  $h^C$ ) was used. In total, 12 samples (2  
680 colours [Black/Red]  $\times$  2 developmental stages [24h AP/72h AP]  $\times$  3 biological replicates)  
681 were prepared for RNA-seq analysis. Two fragments of bored epidermis from left and right  
682 elytra were collected as a single sample in each condition. All total RNA extracted from each

683 sample (12 ng to 158 ng) using RNeasy Mini Kit (QIAGEN) and QIAcube (QIAGEN) was  
684 used for each cDNA library preparation. RNA-seq library preparation was performed using  
685 the SureSelect strand specific RNA library prep kit (Agilent) according to the manufacturer's  
686 instructions. Briefly, mRNA was purified using Oligo-dT Microparticles. The strand-specific  
687 RNA-seq libraries were prepared using dUTP and Uracil-DNA-Glycosylase. The libraries  
688 and its intermediates were purified and size-fractionated by AMPure XP (Beckman Coulter).

689 For quality check and quantification of the RNA-seq libraries, we employed 2100  
690 Bioanalyser and DNA 7500 kit (Agilent). 100 bp paired end read RNA-seq tags were  
691 generated using the Hiseq 2500 (Illumina) following the methods in the User Guide.

692 In advance of reference mapping, adaptor and poly-A sequences were trimmed  
693 from raw RNA-seq reads by using Cutadapt (ver. 1.9.1)<sup>69</sup>. Low-quality reads were also

694 filtered out by a custom Perl script as described previously<sup>83</sup>. The preprocessed RNA-seq  
695 reads were mapped to the reference *H. axyridis* genome sequences using TopHat2<sup>84</sup> (ver.  
696 2.1.0) with default parameters, and assembled by Cufflinks<sup>85</sup> (ver. 2.2.1) with the -u option in  
697 each sample. All predicted transcript units and all loci from different samples were merged  
698 by Cuffmerge in the Cufflinks suite. The RNA-seq read pairs (fragments) mapped to each  
699 predicted transcript unit and locus were counted using HTSeq<sup>86</sup> (ver. 0.6.1) with the options  
700 '-s no -t exon -i transcript' and '-s no -t exon -i locus', respectively. The downstream statistic  
701 analyses were performed using edgeR<sup>87, 88</sup> package (ver. 3.16.5). The raw RNA-seq fragment  
702 counts were normalised by the trimmed mean of M-values (TMM) method. Fold change  
703 between black and red regions in each stage and its statistical significance (FDR) were

704 calculated. The mean fold changes of the genes in the scaffolds including the *h* locus

705 candidate region were visualised with IGV<sup>89, 90</sup> software (ver. 2.3.88).

706

707 **Comparison of the sizes of the upper noncoding regions of *pannier* among**

708 **holometabolous insects**

709 The holometabolous insects, whose genomic sequences are well assembled at the

710 *pannier* locus to the extent that at least the two paralogous GATA transcription factor genes,

711 *GATAe* and *serpent*, are included in the same scaffold, were selected for comparison.

712 Concerning Coleoptera, genomic sequences were collected from the Genome

713 database at NCBI<sup>91</sup> (GCA\_000002335.3, Tcas5.2; GCA\_001937115.1, Atum\_1.0;

714 GCA\_000390285.2, Agla\_2.0; GCA\_000648695.2, Otau\_2.0; GCA\_001412225.1,

715 Nicve\_v1.0; GCF\_000699045.1, Apla\_1.0; GCA\_002278615.1, Pchal\_1.0) and  
716 Fireflybase<sup>92</sup> (*Photinus pyralis* genome 1.3, *Aquatica lateralis* genome 1.3). Concerning  
717 holometabolous insect other than Coleoptera, genomic information at Hymenoptera Genome  
718 Database<sup>93</sup> (Hymenoptera) (GCF\_000002195.4, Amel\_4.5; GCF\_000217595.1,  
719 Lhum\_UMD\_V04; GCF\_000002325.3, Nvit\_2.1), Lepbase<sup>94</sup> (Lepidoptera, butterfly;  
720 *Danaus plexippus*\_v3\_scaffolds), SilkBase<sup>95</sup> (Lepidoptera, silk moth; Genome assembly  
721 [Jan.2017]), Flybase<sup>96</sup> (Diptera, *Drosophila*, dmel\_r6.12\_FB2016\_04), and the Genome  
722 database at NCBI<sup>91</sup> (Diptera, mosquitos; GCA\_000005575.1, AgamP3; GCA\_002204515.1,  
723 AaegL5.0) (Lepidoptera, moth; GCA\_002192655.1, ASM219265v1) were utilised. We  
724 performed BLAST<sup>47</sup> search (TBLASTN, ver. 2.2.26) using the amino acid sequence of *H.*  
725 *axyridis* Pannier as a query, and identified *pannier* orthologs by focusing on the top hits, and



726 the conserved synteny of the three paralogous GATA transcription factor genes, in which  
727 *serpent*, *GATAe*, and *pannier* are tandemly aligned in this order from the 5' to 3' direction.  
728 The sizes of the upper noncoding region of *pannier* were estimated by calculating the  
729 difference between the coordinates of the 3' end of BLAST hit region of *GATAe* and the 5' hit  
730 region of *pannier*. Traces of translocation or insertions between the paralogous GATA genes  
731 were surveyed by looking into the annotations between the *GATAe* and *pannier* loci. If a  
732 noncoding exon 1 was annotated at the *pannier* locus, the size of the first intron was also  
733 calculated as well.

734

735 **Motif enrichment analysis of the upper noncoding regions of *pannier***

736 To search for the *Drosophila* known DNA-binding sites at *pannier*, 1,139 DNA  
737 motifs were retrieved from the JASPAR<sup>97</sup> database using the MotifDb<sup>98</sup> R package.  
738 Concerning the SD-binding motif, the position weight matrix (PWM) scores were calculated  
739 using the 2,557 ChIP-seq peaks in *Drosophila* genome obtained by Ikmi et al.<sup>99</sup> and the  
740 RSAT peak-motifs programme<sup>100</sup>. The nucleotide sequences at the three upper noncoding  
741 regions of *pannier* (the upper intergenic region, the upstream half of the 1st intron, and the  
742 down stream half of the 1st intron) were collected by forging BSgenome<sup>101</sup> data packages  
743 using our *H. axyridis* and *C. septempunctata* genome sequences, and by retrieving the  
744 sequences using coordinate information obtained for annotation in Fig. 5a and the  
745 GenomicFeatures<sup>102</sup> R package. We here defined the upstream half of the 1st intron as the  
746 region including all of the traces of inversions or corresponding sequences shared among

747 different *h* alleles in *H. axyridis*. GRange objects were generated using the coordinate  
748 information obtained for annotation in Fig. 5a. Motif enrichment was quantified using the  
749 PWMEnrich<sup>103</sup> R package. As control background genomic regions for the upper intergenic  
750 regions of *pannier*, we used 2 kb promoter sequences of 11,279 genes to which RNA-seq  
751 reads were mapped (*H. axyridis* genome assembly version 1), and not located within the 10  
752 kb from the end of the genomic scaffolds. As control background genomic regions for the  
753 upstream and downstream regions of the 1st intron of *pannier*, we used 2 kb sequences at the  
754 5' and 3' end of the 1st introns, which are more than 2 kb long, and without gaps (2825 and  
755 2810 sequences, respectively). Since there is no reliable gene annotation data for the *C.*  
756 *septempunctata* genome, we used the *H. axyridis* genomic background sequences. Each  
757 motif enrichment score, which is related to average time that transcription factors spend in

758 binding to a DNA sequence<sup>104</sup>, was calculated using default parameter of PMWEnrichment.

759 *br* and *da* DNA-binding motifs were excluded from the analysis.

760

### 761 **Molecular phylogenetic analysis**

762           Concerning the conserved regions in the upper half of the 1st intron of *pannier*,

763 nucleotide sequences were collected from the BLAST hits obtained to construct Fig. 5a. The

764 collected BLAST hit sequences were arranged in the same directions using a custom Perl

765 script, and aligned using MAFFT<sup>105</sup> (ver. 7.222). We concatenated three alignment blocks,

766 and manually excluded GAP sites and seemingly nonhomologous sites in the alignment

767 (Supplementary Data 1). Concerning the coding region, nucleotide sequences of the cloned

768 cDNAs were aligned, and trimmed in the same way (Supplementary Data 1).

769           The maximum-likelihood (ML) phylogenetic trees were constructed using  
770   RAxML<sup>106</sup> (ver. 8.0.0) with the options '--maxiterate 1000 --localpair --clustalout'. We  
771   determined appropriate models of sequence evolution under the AICc4 criterion using  
772   Kakusan4<sup>107</sup>. 100 replicates of shotgun search for the likelihood ratchet were performed.  
773   Nodal support was calculated by bootstrap analyses with 1000 replications.

774

775

776   **Data availability**

777           The DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory  
778   (EMBL)/GenBank accession numbers for the sequences reported in this article are  
779   LC269047–LC269055 for *Ha-pnr*, LC269056 for *Cs-pnr* and LC269057 for *Cs-rp49*. The

780 genomic sequencing data, resequencing data and the RNA-seq data were deposited in the  
781 DNA Data Bank of Japan (DDBJ) under the accession number DRA002559, X (under  
782 curation) & DRA006068, and DRA005777, respectively.

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989 **Supplementary Information**

990 Below are supplementary data related to our analyses. Included are one table on our  
991 small-scale RNAi screen, one table on statistics of genome assemblies, five tables on  
992 DNA primers designed for amplification and sequencing, and five figure legends for the  
993 Supplementary data.



994

Supplementary Table 1 | The 1st small-scale RNAi screening of wing/body wall patterning genes

gene name	phenotype in elytra	tested alleles
<i>araucan</i>	no phenotype	<i>h, h<sup>C</sup>, h<sup>Sp</sup>, h<sup>A</sup></i>
<i>aristaless</i>	no phenotype	<i>h</i>
<i>apterous</i>	wrinkled elytra	<i>h<sup>Sp</sup>, h<sup>A</sup></i>
<i>blistered</i>	lethal	<i>h<sup>Sp</sup>, h<sup>A</sup></i>
<i>Cubitus interruptus</i>	pupal lethal	<i>h<sup>A</sup></i>
<i>Distal-less</i>	no phenotype	<i>h, h<sup>C</sup></i>
<i>decapentaplegic</i>	smaller elytra	<i>h, h<sup>A</sup></i>
<i>Epidermal growth factor receptor</i>	lethal	<i>h<sup>Sp</sup>, h<sup>A</sup></i>
<i>pannier</i>	transformation of black regions to red regions	<i>h, h<sup>C</sup>, h<sup>Sp</sup>, h<sup>A</sup></i>
<i>wingless</i>	no phenotype	<i>h, h<sup>Sp</sup></i>

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Marker/primer name	Target	Sequence (5'>3')	polymorphism (coordinate) between h-F0 and hC-F0, between hA-F0 and hSp-F0, and between hA-F0 and hC-F0	Recombinants' genotype	Cross	The <i>h</i> candidate regions	
mbi-F	scaffold898_cov129:657,001-657,297	AAAATGAGAGTGTAAAGAAGA	A/G (657,138)	h/h-#27 (A/G heterozygote) hC/hC-#1 (A/G heterozygote)	$h \times h^C$ $h \times h^C$	< <i>h</i>	< $h^C$
mbi-R		CAAAGCAGAGAAACCTAACA					
DUS-F	scaffold898_cov129:1,560,979-1,561,277	CATTTCAACATCATCTTTCTG	A/T (1,561,052)	no recombinant	$h \times h^C$ $h \times h^C$	<i>h</i>	$h^C$
DUS-R		GAGACGGAGAGAGTGAAC					
pnr-F	scaffold294_cov134:640,762-640,221	AAATTATTCTTACACAATTTCTGG	600bp/500_or_480bp	no recombinant	$h \times h^C$ $h \times h^C$	<i>h</i>	$h^C$
pnr-R		AGGACACGTTTGAAGAATAAGATAGT					
mus201-F	scaffold267_cov168:390,452-390,521	CCAAGAGGCTCAGGTAATA	70bp/100bp	hC/hC-#14 (70bp/100bp heterozygote)	$h \times h^C$ $h \times h^C$	<i>h</i>	$h^C >$
mus201-R		ATTTCCATACAACACCAAAT					
Mink-F	scaffold99874_cov139:62,450-62,532	GAGTCTCCAAAATTACCAT	80bp/80_or_57bp	h/hC-#7 (57bp homozygote)	$h \times h^C$ $h \times h^C$	<i>h &gt;</i>	
Mink-R		CAATGCTCTTCTGAAATAA					
Fam92_3'-F	scaffold294:205,919-205,999	GTCTGTCACTTCTAGYGTATATGAAT	80bp/~60bp	hA/hSp-#41 (~60bp homozygote) hA/hSp-#49 (~60bp homozygote)	$h^A \times h^{Sp}$ $h^A \times h^{Sp}$	< $h^A$	< $h^{Sp}$
Fam92_3'-R		AGTTCGGTCAAGCAATTG					
sens-F	scaffold294:309,273_309,615	TCTTCATGGAGAGGTTTA	242bp/~250bp	no recombinant	$h^A \times h^{Sp}$ $h^A \times h^{Sp}$	$h^A$	$h^{Sp}$
sens-R		GMGTTTGTTCAAATTCATA					
srp_3'-F	scaffold267:650,101-650,598	ACTTTAACAACCCCGACR	~170bp/498bp	no recombinant	$h^A \times h^{Sp}$ $h^A \times h^{Sp}$	$h^A$	$h^{Sp}$
srp_3'-R		TACCATATTGAATCAACGTT					
srp_5'-F	scaffold267:579,180-579,337	AATAAGCATAAACTTCCT	157bp/~160bp	hSp/hSp-#61 (157bp/~160bp heterozygote)	$h^A \times h^{Sp}$ $h^A \times h^{Sp}$	$h^A$	$h^{Sp} >$
srp_5'-R		ACTCACAAATAGGTTTCTGAA					
kis_5'-F	scaffold267:465,373-465,708	AGCAATTTGTATGTATTGAG	~290bp/335bp	hSp/hSp-#61 (~290bp/335bp heterozygote)	$h^A \times h^{Sp}$ $h^A \times h^{Sp}$	$h^A$	
kis_5'-R		ATGTGAATTAACAAGGTG					
kis_3'-F	scaffold267:421,269-421,340	TTTTCTCGYTRGTATCAA	~60bp/71bp	hSp/hSp-#61 (~60bp/71bp heterozygote) hA/hSp-#9 (71bp homozygote)	$h^A \times h^{Sp}$ $h^A \times h^{Sp}$	$h^A >$	
kis_3'-R		CAAAATTTGAMACATGTTT					
RAD-tag_5'_breakpoint	scaffold294:466,936	- -	C/- (466,936)	hC/hC-#56 (C/- heterozygote) hA/hC-#1 (-/- homozygote)	$h^A \times h^C$ $h^A \times h^C$	< $h^A$	< $h^C$

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**Supplementary Table 3 | Statistics of the genome assemblies**

Columns labelled as “Complete”, “Duplicated & complete”, “Fragmented” and “Missing” are the results from the BUSCO programme.

Speceis	Sample	Assembler	Input data	Total (bp)	No. of sequences	N50 length (bp)	Gap rate (%)	Max length (bp)	Complete (%)	Duplicated & complete (%)	Fragmented (%)	Missing (%)
<i>H. axyridis</i>	<i>h<sup>c</sup></i> F2-3	Platanus2	pair end & mate pair reads	434,974,256	18,513	1,106,177	4.62	10,486,871	97.587	2.714	1.267	1.146
<i>H. axyridis</i>	<i>h<sup>c</sup></i> NT6	Supernova	10x linked-reads	581,452,270	50,767	100,132	10.24	8,301,291	95.476	9.288	2.654	1.87
<i>H. axyridis</i>	<i>h<sup>A</sup></i> F2 hybrid	Supernova	10x linked-reads	510,576,820	50,277	57,217	4.5	6,058,326	95.838	7.539	2.593	1.568
<i>H. axyridis</i>	<i>h</i> NT8	Supernova	10x linked-reads	557,776,217	44,316	157,105	11.07	15,325,500	95.356	7.358	2.774	1.87
<i>C. septempunctata</i>	MD-4	Supernova	10x linked-reads	514,983,385	55,574	67,752	9.12	12,752,393	96.019	5.79	2.292	1.689

1002 **Supplementary Table 6 | Primers for gene cloning**

Primer name	Target direction	Sequence (5'>3')
pannier-F	sense	ATIGAYTTYCARTTYGGIGA
pannier-R	antisense	GGYTTICKYTTICKIGTYTG
rp49-F	sense	ACIAARMAITTYATIMGICA
rp49-R	antisense	TGIGCIATYTCISRCARTA
Ha-pannier-RACE-1	sense	GGGCAGGGAGTGCGTCAATTGTGGGCC
Ha-pannier-RACE-2	sense	CCACCCCTCTGTGGAGGAGAGATGGTAC
Ha-pannier-RACE-3	antisense	GCCACAGGCGTTGCACACCGTTGCGCC
Ha-pannier-RACE-4	antisense	GATGCCGTCCTTGCGCATGGCCAGGGG
Cs-pannier-RACE-1	sense	AATTGCGGCACCAGGACGACGACGCTC
Cs-pannier-RACE-2	sense	AAGCTGCACGGCGTCAACAGGCCTCTG
Cs-pannier-RACE-3	antisense	ATCATCATGTTCTGGGCGTA
Cs-pannier-RACE-4	antisense	GTACGGGCTAAGTTCGGATC
Cs-pannier-5	sense	GATCCGAACTTAGCCCGTAC
Cs-pannier-6	antisense	GGGTCTGTTATCCCATTCATCTTGTGG
Ha_pnr_intron_conserved1_F	sense	TCAGCRAATCTTCACATA
Ha_pnr_intron_conserved1_R	antisense	CTCCACTCGTTTATCTTAAT
Ha_pnr_intron_conserved2_F	sense	AGAGAAAAGAGACAASTTGA
Ha_pnr_intron_conserved2_R	antisense	AAAAGTRTTT SCTTCAGG
Ha_pnr_intron_conserved3_F	sense	AATGKATTCAAACCYCAGAC
Ha_pnr_intron_conserved3_R	antisense	MGRACGCTGAATGAAAGT

(I=inosine, K=G+T, M=A+C, R=A+G, S=C+G, Y=T+C)

1003 **Supplementary Table 7 | Primers for RT-PCR**

Primer name	Target direction	Sequence (5'>3')
Ha-pannier-1	sense	TCGAGCCTGGTGAAGAGCGAACCGGG
Ha-pannier-2	antisense	GGGTCTCCGACGCAGTATTGATCTC
Ha-pannier-3	sense	GCTCCACCTCCGTAGAAGAC
Ha-pannier-4	antisense	AGCCATCAGTTTGGCAGAAG
Cs-pannier-1	sense	GGCGGTGAACGAGATGACAG
Cs-pannier-2	antisense	GCCATCAGTTTAGCGGACGC
Cs-pannier-3	sense	TGTGGAGGCGGGATGGTACT
Cs-pannier-4	antisense	CTGTTGACCCGTGCAGCTT
Ha-rp49-1	sense	GCGATCGCTATGGAAACTC
Ha-rp49-2	antisense	TACGATTTTGCATCAACAGT
Cs-rp49-1	sense	AGTGATCGTTATGGCAAGCT
Cs-rp49-2	antisense	TCTGTTTTGCATCAAAGGAC

1004 **Supplementary Table 8 | Primers for direct sequencing of *Ha-pannier* ORF**

Primer name	Target direction	Sequence (5'>3')	Use of primer
Ha-pannier-ORFa-F	sense	GCCACTGTCCGTAATTAGCCCGAACAGG	PCR-1
Ha-pannier-ORFa-R	antisense	TCCACCAGAAATAAGGAAATGAGG	PCR-1
Ha-pannier-seq1	sense	GGGCAGGGAGTGCCTCAATTGTGGGGCC	Sequencing-1
Ha-pannier-seq2	antisense	GGTCTGTTCATCCCATTATCTTGTGG	Sequencing-1
Ha-pannier-seq3	sense	AAAACAAGGTGGTGGTAGT	Sequencing-1
Ha-pannier-seq4	antisense	ATAAGGTGACGTCGGTTGGAATCCAGA	Sequencing-1
Ha-pannier-seq5	sense	CCACAAGATGAATGGGATGAACAGACCC	Sequencing-1
Ha-pannier-seq6	antisense	TCTGTCTTGTATGTCGT	Sequencing-1
Ha-pannier-5'-F	sense	AGTTCTCCAAGCCCTCAAAGTTCAACGAC	PCR-2
Ha-pannier-5'-R	antisense	GGTCTGTTCATCCCATTATCTTGTGG	PCR-2
Ha-pannier-seq7	sense	CATCGTCTCAGATTAGGTGTAACGACG	Sequencing-2
Ha-pannier-seq8	antisense	ATAAGGTGACGTCGGTTGGAATCCAGA	Sequencing-2
Ha-pannier-exon3B-F1	sense	GTTCCACCAACACCTTC	PCR-3
Ha-pannier-exon3B-R1	antisense	GTCTGGTTGCAGTTAGTATT	PCR-3
Ha-pannier-seq9	antisense	GATGCCGTCCTTGCCATGGCCAGGGG	Sequencing-3
Ha-pannier-exon3B-F2	sense	ACCTTATGAACATGGATAC	PCR-4
Ha-pannier-exon3B-R2	antisense	GATATGCTTACTGGTGTCT	PCR-4
Ha-pannier-seq10	sense	TCTCCCCTCTCCGCCGGTCAGTTCTAC	Sequencing-4

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**Supplementary Table 9 | Primers for riboprobe synthesis (*in vitro* transcription, IVT)**

Primer name	Target direction	Sequence (5'>3')	Use of primers
Ha-pannier-ORF-F	sense	GTCAGCATGTTCCACACC	ORF cloning
Ha-pannier-ORF-R	antisense	TCTTGTCTTGTATTATGTCGT	ORF cloning
T3	sense	ATTAACCCTCACTAAAGGGA	IVT template (5' ORF)
SP6-Ha-pnr-NR	antisense	atttagtgacactatagaACTCCATGGCACTCTC	IVT template (5' ORF)
T7	antisense	TAATACGACTCACTATAGGG	IVT template (3' ORF)
SP6-Ha-pnr-CF	sense	atttagtgacactatagaAAACAAGGTGGTGGTAGT	IVT template (3' ORF)

1006 **Supplementary Figure 1 | Snapshots of pharate adult elytral pigmentation in *H.***

1007 *axyridis*

1008 Melanin synthesis activity (black) and carotenoids accumulation (orange) were

1009 simultaneously visualised using  $h^C$  elytra stained with PO activity. Strong black and

1010 orange signals appeared after 80 h AP.

1011

1012 **Supplementary Figure 2 | Expression analysis of *H. axyridis* (*Ha*) *pannier* in elytral**

1013 **primordia by RT-PCR.**

1014 **a**, Developmental expression profiles of *pannier* from the final instar larvae to pupae.

1015 *Ha-pnr*, *Ha-pannier*, *Ha-rp49*, *Ha-ribosomal protein 49* (internal control). Adult

1016 emergence is at 4.5 days AP in our rearing condition. Days after the onset of the each

1017 stage are indicated above. **(b, b')** Spatial distribution of *pannier* at 84 h AP. Future black

1018 (B1-B3) and red (R) regions were isolated for RT-PCR. Pharate adult elytra of three

1019 individuals were analysed (#1-#3). *rp49*, internal control. **b'**, Left panel, pharate adult

1020 elytra with 3 hour PO staining (84 h AP). Right panel, an example of an elytron after

1021 isolation of red and black regions. Scale bars, 1 mm.



1022

1023 **Supplementary Figure 3 | Validation of the *pannier*-locus scaffold of Platanus2 for**  
1024 **the *H. axyridis* F2-3 sample**

1025 The scaffold was segmented into 2 kbp-windows, and links of 15 kbp-mate-pairs between  
1026 windows (the number of mate-pairs > 2) were visualised as arcs .The entire region of the  
1027 scaffold was uniformly covered by the mate-pairs, and no links between a distal  
1028 window-pair inferring mis-assembly was observed.

1029

1030 **Supplementary Figure 4 | Dot plots of the genomic scaffolds including the *pannier***  
1031 **locus**

1032 **a–d**, The dot plots between the consensus scaffolds obtained by reassembly of F2-3  
1033 mate-pair reads with Platanus2, and each consensus scaffold obtained by *de novo* genome  
1034 assembly of the 10x linked-reads. **a**,  $h^C$  (F2-3) vs.  $h^C$  (NT6). **b**,  $h^C$  (F2-3) vs.  $h^A$   
1035 (F2-hybrid). **c**,  $h^C$  (F2-3) vs.  $h$  (NT8). **d**,  $h^C$  (F2-3) vs. *C. sep*. The colour code for  
1036 colouring the homologous segments is on the right side of each panel. Blue arrow, the  
1037 *pannier* locus. The green and the magenta pins indicate the position of the breakpoint

1038 genotyping markers located on the outermost side, of all genotyping markers found to  
1039 show association with the *h* locus in the three crossing experiments (*mb1* and *Mink* in  
1040 Supplementary Table 2, respectively). The subset regions of each scaffold between these  
1041 two markers or the corresponding regions were extracted for dot plot analyses. In each  
1042 pair of comparison, sequential linear homology between the two scaffolds was confirmed.  
1043 The responsible region of each *h* allele ( $h^C$ , 690 kb;  $h^A$ , 660 kb;  $h$ , 2.1 Mb +  $\alpha$ ) was  
1044 included in a single linked-read genomic scaffold (a–c). In *C. septempunctata*, the  
1045 genomic region corresponding to the region just downstream of the *pannier* locus in *H.*  
1046 *axyridis*, was located in the scaffold different from that including *pannier* (scaffold 47  
1047 and 92), implying at least one translocation event in either of the two ancestral lineages  
1048 **(d)**.

1049

1050 **Supplementary Figure 5 | Polymorphism in ORF sequences of *pannier-A* isoform in**  
1051 **the 4 allelic strains of the *h* locus.**

1052 **a**, Nucleotide polymorphisms in *pannier* ORF in 4 allelic strains of *h* ( $h$ ,  $h^A$ ,  $h^{Sp}$ ,  $h^C$ ). R =

1053 G or A, Y = T or C, M = A or C, S = G or C, W = A or T. **b**, Amino acid sequences deduced

1054 from ORF sequences in (a). X = T or S. The alternative exon region (exon 3A) and GATA

1055 zinc finger domains are indicated with grey and yellow, respectively.

1056

1057 **Supplementary Figure 6 | Polymorphism in ORF sequences of *pannier-B* isoform in**

1058 **the 4 allelic strains of the *h* locus.**

1059 **a**, Nucleotide polymorphisms in *pannier* ORF in 4 allelic strains of *h* (*h*, *h<sup>A</sup>*, *h<sup>Sp</sup>*, *h<sup>C</sup>*). R =

1060 G or A, Y = T or C, M = A or C, S = G or C, W = A or T. **b**, Amino acid sequences deduced

1061 from ORF sequences in (a). X = T or S. The alternative exon region (exon 3B) and GATA

1062 zinc finger domains are indicated with grey and yellow, respectively.

1063

1064 **Supplementary Figure 7 | *vestigial* mRNA is upregulated in the presumptive black**

1065 **regions from early pupal stages in the *h<sup>C</sup>* background.**

1066 1B, black region at 24 h AP. 1R, red region at 24 h AP. 3B, black region at 72 h AP. 3R,

1067 red region at 72 h AP. Read count of each sample was extracted from RNA-seq analysis

1068 data. Bars, mean read counts. (n = 3). Error bars, standard error of means. \*, FDR < 0.01.

1069

1070 **Supplementary Figure 8 | Expression analysis of *C. septempunctata* (*Cs*) *pannier* in**

1071 **elytral primordia by RT-PCR.**

1072 **a**, Developmental expression profiles of *pannier* in pupal stages. *Cs-pnr*, *Cs-pannier*.

1073 *Cs-rp49*, *Cs-ribosomal protein 49* (internal control). Adult emergence is at 4.5 days AP in

1074 our rearing condition. Days after the onset of the each stage are indicated above. **b**, **b'**,

1075 Spatial distribution of *pannier* at 84 h AP. Future black (B1–B2) and red (R1–R3) regions

1076 were isolated for RT-PCR. Pharate adult elytra of three individuals were analysed

1077 (#1–#3). *Cs-rp49*, internal control. **b'**, Left panel, pharate adult elytra with 3 hour PO

1078 staining (84 h AP). Right panel, an example of an elytron after PO staining for 1 hour and

1079 isolation of red and black regions. Scale bars, 1 mm.

1080

1081 **Supplementary Data 1 | DNA sequences used in the molecular phylogenetic analyses**

1082 **a**, The aligned nucleotide sequences of *pannier* ORF before trimming gapped and shifted

1083 regions. **b**, The aligned nucleotide sequences of *pannier* ORF after trimming. **c**, The

1084 aligned nucleotide sequences of the conserved intronic regions of *pannier* (concatenated

1085 three blocks) before trimming gapped and shifted regions. **d**, The aligned nucleotide

1086 sequences of the conserved intronic regions of pannier after trimming. A dash indicates a

1087 gap.

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1101 **Author contributions**

1102 T.N. and T.A conceived this study. T.M. and T.N. analysed the elytral  
1103 pigmentation processes. T.A., T.M., K.G., K.H., A.I. and J.Y. analysed the sequence data.  
1104 T.A, T.M., K.G., A.I., K.H., and J.H. performed cloning of the *pannier* genes from  
1105 different alleles and species of ladybirds. T.M., K.G., A.I., K.H., and J.H. performed the  
1106 larval RNAi experiments. K.H., K.G. and J.H. performed the semi-quantitative RT-PCR.  
1107 T.A. performed the *in situ* hybridisation. J.Y. collected the total RNA for the RNA-seq  
1108 analysis, and the genomic DNA samples for the initial *de novo* genome assembly. T.A.  
1109 collected the DNA samples for the resequencing analyses. M.S. and Y.S. collected the  
1110 RNA-seq raw data. Y.M. and A.T. performed the initial *de novo* genome assembly. K.Y.  
1111 and S.S. collected the raw data for the resequencing and the RAD-seq analyses. K.Y.  
1112 constructed the flexible ddRAD-seq protocol. R.K, M.O, and T.I. performed reassembly  
1113 of the genome, *de novo* assembly of the linked-read genomic data, and validation of the  
1114 obtained genomic scaffolds. M.K., T.T., T.A. and K.Y. performed mapping and  
1115 quantification of the RNA-seq data. T.A. performed the data analyses for the genetic  
1116 association studies, the gene annotation, the motif enrichment analysis, and the molecular

1117 phylogenetic analyses around the *pannier* locus. T.A and T.N. wrote, and all authors

1118 commented on the manuscript.

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1124 DRA006068 for the genomic resequencing data of *H. axyridis*, and DRA005777 for the  
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