Title

Repeated inversions at the *pannier* intron drive diversification of intraspecific colour patterns of ladybird beetles
Abstract

How genetic information is modified to generate phenotypic variation within a species is one of the central questions in evolutionary biology. Here we focus on the striking intraspecific diversity of more than 200 aposematic elytral (forewing) colour patterns of the multicoloured Asian ladybird beetle, *Harmonia axyridis*, which is regulated by a tightly linked genetic locus *h*. Our loss-of-function analyses, genetic association studies, *de novo* genome assemblies, and gene expression data reveal that the GATA transcription factor gene *pannier* is the major regulatory gene located at the *h* locus, and suggest that repeated inversions and cis-regulatory modifications at *pannier* led to the expansion of colour pattern variation in *H. axyridis*. Moreover, we show that the colour patterning function of *pannier* is conserved in the seven spotted ladybird beetle, *Coccinella septempunctata*, suggesting that *H.*
axyridis’ extraordinary intra-specific variation may have arisen from ancient modifications in a conserved elytral colour patterning mechanisms in ladybird beetles.
There are approximately 6,000 ladybird beetle species described worldwide¹. Charismatic and popular, ladybird beetles are famous for the red and black spot patterns on their elytra (forewings), thought to be a warning signal to predators that they store bitter alkaloids in their body fluids²,³ and are unpalatable. This red/black warning signal is shared among many ladybird beetle species, and provides a model for colour pattern mimicry by other insect orders. While most ladybird beetle species have only a single spot pattern, a few display remarkable intraspecific diversities, such as the multicoloured Asian ladybird beetle, *Harmonia axyridis*, which exhibits more than 200 different elytral colour forms (Fig.1a). This striking intraspecific variation prompted us to investigate its genetic and evolutionary basis.
The first predictions regarding the genetics underlying the highly diverse elytral
colour patterns of *H. axyridis* were made by the evolutionary biologist, Theodosius
Dobzhansky based on his comprehensive classification of specimens collected from various
regions in Asia. Successive genetic analyses revealed that many of these colour patterns
are actually regulated by a tightly linked genetic locus, *h*, which segregates either as a single
gene, or as strongly linked pseudoallelic genes (a super-gene) (Fig.1b, c). The elytral
colour patterns are assumed to be formed by the superposition of combinations of two of the
four major allelic patterns and dozens of minor allelic colour patterns (more than 20 different
allelic patterns in total). The major allelic patterns cover more than 95% of colour patterns in
the natural population. In the elytral regions where the different colour elements are
overlapped in heterozygotes, black colour elements are invariably dominant against red
colour elements (mosaic dominance\textsuperscript{10}). Whether all of the supposed alleles linked to the $h$
locus correspond to a single gene or multiple genes is yet unknown. Elucidating the DNA
structure and the mechanisms underlying the evolution of this tightly linked genetic locus
that encodes such a strikingly diverse intraspecific colour pattern polymorphism would
provide a case-study that bears upon a major evolutionary-developmental biology question;
how does morphology evolve? Here we show that the gene \textit{pannier} is responsible for
controlling the major four elytral colour patterns of \textit{H. axyridis}. Moreover, we illustrate how
modification to this ancient colour-patterning gene likely contributed to an explosive
diversification of colour forms.
Figure 1. Intraspecific genetic polymorphisms of elytral colour patterns in *Harmonia axyridis*. a, Highly diverse elytral colour patterns of *H. axyridis*. b, Four major alleles of the elytral colour patterns: *h^C*, *h^Sp*, *h^A*, *h*, *h^s*. c, An example of inheritance of elytral colour forms. When *h^C/h^C* and *h/h* are crossed (P), all F1 progenies show the colour pattern of *h^C/h*. Note the small black spots within the red spots. When the F1 heterozygotes are sib-crossed, F2 progeny show three phenotypes (*h^C/h^C*, *h^C/h*, and *h/h*) at the 1:2:1 ratio predicted for Mendelian segregation of a single locus. Inheritance of any combination of colour patterns follows this segregation pattern.
**Results**

To identify the gene regulating elytral colour pattern formation of *H. axyridis*, we first investigated the pigmentation processes during development. In the developing pupal elytra, red pigment (carotenoids) was accumulated in the future red-pigmented regions (Fig. 2a pharate adult elytron). Red pigmentation occurred only in the thick ventral epidermal cells of the two layers of the elytral epidermis (Fig. 2b; 2c, Red), and started at 80 hours after pupation (80 h AP). Black pigmentation (melanin accumulation) occurred only in the dorsal cuticle of black-pigmented regions (Fig. 2d), and started approximately 2 h after eclosion.

Although pharate adult elytra are not black, we detected a strong upregulation of enzymatic activity related to melanin synthesis in the nascent dorsal cuticle in the future black regions from 80 h AP (Fig. 2a, lower panels; Fig. 2c, black; Supplementary Figure 1). Every
black-pigmented region was deployed complementary to the red regions. Therefore, we concluded that the developmental programmes for both red and black pigmentation started around 80 h AP.
Figure 2 | Developmental programmes for elytral pigmentation initiate at the late pupal stage in *H. axyridis*. **a.** Adult elytral colour patterns (upper panels), and localisation of carotenoid (middle panels, orange) and phenol oxidase (PO) activity (lower panels, black) in pharate adult elytra at 96 h AP. Proximal is up. Outer rims are to the left. **b–d.** Cross sections of elytra at 96 h AP (**b, c**) and adult (**d**). Dorsal is up. **b,** magenta, nuclei (propidium iodide); green, F-actin (phalloidin). **c,** left, PO staining; right, No staining. Arrowheads indicate pigmented areas. **d,** Haematoxylin and eosin stain. Scale bars, 1 mm in (**a**), 50 μm in (**b, c, d**).
We hypothesised that some of the conserved genes essential for insect wing/body wall patterning are recruited to regulate these elytral pigmentation processes, and tested this possibility using larval RNAi. We performed small-scale candidate screening focusing on genes involved in wing/body wall patterning (Supplementary Table 1), and found that the Harmonia ortholog of Drosophila panner, which encodes a GATA transcription factor, is essential for formation of all of the black-pigmented regions in the elytra. For all four major h allele backgrounds, larval RNAi targeting panner resulted in complete loss of black colour elements and alternative emergence of red colour elements in the elytra (Fig. 3a, H. axyridis), indicating that panner is essential for inducing black pigmentation in dorsal elytral cells and suppressing red pigmentation in ventral elytral cells. This result was unexpected because panner is not essential for wing blade patterning in Drosophila, but rather essential for
patterning of the dorsal body plate attached to the wings (notum) \(^{21-23}\).
Figure 3 | The pattern of pannier expression foreshadowing the adult elytral colour pattern is necessary for switching red/black pigmentation processes in ladybird elytra.

a. The adult phenotypes of RNAi treatments targeting GFP (negative controls, GFP RNAi) and pannier (pnr RNAi) in *H. axyridis* (h\(^c\), h\(^w\), h\(^a\), h) and *C. septempunctata*. Scores in the lower left corners indicate penetrance of the loss-of-pattern phenotype in surviving animals.

b. The pattern of pannier expression (pnr) in the dorsal elytral epidermal cells immediately before or after pigmentation (76–84 h AP). Left panels indicate the corresponding adult elytral phenotypes (h\(^c\) and h). White arrowhead, the region with a weak signal. Black arrowheads, the regions with intense signals. Scale bars, 1 mm.
pannier mRNA was upregulated from 48 h AP to 96 h AP in elytra, and preferentially in black regions ($h^c$, Supplementary Figure 2). Immediately before or after 80 h AP (start of the pigmentation programme, 76–84 h AP), pannier seemingly showed higher expression in the future black regions in the dorsal elytral epidermis (Fig. 3b). These data suggest that region-specific upregulation of pannier during the pupal stage regulates black pigmentation in the ladybird beetle’s dorsal elytral cells, and that regions of expression differ among the major $h$ alleles to form different black patterns in $H. axyridis$.

These data led us to test whether pannier is associated with the classically identified locus $h$, which regulates elytral colour patterns. To identify DNA sequences near the $h$ locus, we assembled de novo genome sequences (assembly version 1: 423 Mb; contig N50, 63.5 kb; scaffold N50, 1.6 Mb), and performed a genetic association study using the
strains with different \( h \) alleles. We obtained the scaffold containing pannier and two additional adjacent scaffolds based on the truncated gene structures at the scaffold ends (Fig. 4a, Bgb and pnr). Restriction-site Associated DNA Sequencing (RAD-seq) analysis of backcrossed progenies (BC1, \( h^A \times h^C \) F0 cross, \( n = 183 \)) revealed that these three scaffolds are included in the five scaffolds that showed complete association with colour patterns (Fig. 4a, the upper left panel). In addition, genotyping of F2 individuals from two other independent genetic sib-crosses (\( h^C \times h \) F0 cross \([ n = 80 ]\) and \( h^A \times h^{Sp} \) F0 cross \([ n = 273 ]\)) indicated that the pannier locus is included in the relevant regions of all of the major four \( h \) alleles (\( h^C \), 690 kb; \( h^{Sp} \), 750 kb; \( h^A \), 660 kb; \( h \), >2.1 Mb) (Fig. 4a, Supplementary Table 2).
Figure 4 | *pannier* is the major elytral colour patterning gene located at the *h* locus.

**a.** Genetic association study of the *h* locus. Upper left panel, LOD plot for 4,419 RAD tags deduced from genotyping BC1 progeny from the *h<sup>C</sup>*-*h<sup>A</sup>* F<sub>0</sub> cross. RAD tags showing segregation patterns of markers located on the X chromosome were excluded from the analysis. The LOD score peaked at RAD tags in Linkage Group 2. RAD tags with complete association with elytral colour patterns corresponded to 5 genomic scaffolds including the three scaffolds adjacent to the *pannier* locus (lower bars). Upper right panel, the candidate genomic regions responsible for the major four *h* alleles. Lower bars, the three genomic scaffolds adjacent to the *pannier* locus. Grey, genes predicted from RNA-seq. Green, the *pannier* locus. Contiguity of the scaffolds was predicted based on the truncated genes at the end of scaffolds (*Bgb* and *pnr [pannier]*). Arrows indicate the respective candidate genomic regions responsible for each allele. *pannier* is included in all four relevant regions. **b.** Fold changes of gene expression in the presumptive red and black elytral epidermis before pigmentation around the candidate genomic region responsible for *h*. Grey and red bars on the bottom indicate predicted genes. Red, FDR < 0.01. Only *pannier* was significantly upregulated in this region. The samples for RNA-seq were collected as depicted in the right panel in *h<sup>C</sup>* background. **c.** Gene structures of *pannier* in *H. axyridis*. 1A isoform cDNA was cloned by rapid amplification of cDNA ends (RACE). 2A–4B isoforms were predicted from RNA-seq analysis. *pannier* has at least 4 transcription start sites. Coding sequences are located from exon 2 to exon 5 (yellow). There are two alternative exons at exon 3, one encoding one of the two zinc finger domains of Pannier (A isoforms), and the other skipping that zinc finger domain (B isoforms).
To test contiguity of these three scaffolds, we re-assembled the genome using a novel genome assembler (Platanus2), and additional *de novo* genomic assemblies of $h^C$, $h^A$ and $h$ alleles using linked-read and long read sequencing platforms (10x Genomics Chromium system; PacBio system). We obtained longer genomic scaffolds including the three described above ($h^C$, 3.13 Mb/2.74 Mb; $h^A$, 1.42 + 1.61 Mb; $h$, 2.79 Mb, Supplementary Table 3) and the genotyping markers showing complete association with colour patterns and incomplete association at both ends ($h^C$ and $h$) or one end ($h^A$) of each scaffold (Supplementary Figures 3, 4a–c). These data support the result of our genetic association studies.

To further delimit the candidate genes associated with the elytral colour patterns, we performed RNA-seq analysis using epidermal tissues isolated from the developing red or black regions before pigmentation in the $h^C$ genetic background (Fig. 4b, 24 h & 72 h AP).
RNA-seq). We found that *pannier* was the only gene statistically significantly upregulated in the developing black region compared to the red region at 72 h AP within the *h* locus candidate region (Fig. 4b, red bars, False discovery rate [FDR] < 0.01; Supplementary Table 4). These data pinpoint *pannier* as the major gene regulating the elytral colour pattern variation in *H. axyridis*.

We next investigated allele-specific polymorphisms at the *pannier* locus. We found that alleles of the first intronic region of *pannier* are more diverse than the surrounding genomic regions (Fig. 5a, the whitish regions in the middle), whereas the same allele in different strains shows conserved fragments distributed throughout the region (Fig. 5a, blue bars, *hC[F2-3]*–*hC[NT6]*). In comparisons between the alleles, we consistently found traces of large inversions in the upstream half of the first intron (reddish lines in Fig 5a, *hC–hA, hA–h*,
However, we found only a single non-synonymous substitution in the region not conserved among organisms (G235V, hSp), (Supplementary Figure 5, 6), suggesting that cis-regulatory differences in the 1st intronic region of pannier are the major cause of intraspecific colour variation.
Figure 5. Intraspecific diversification and interspecific conservation of the 1st intronic region of *pannier* in ladybird beetles. **a**, Sequence comparison of the genomic region surrounding the *pannier* locus. 700 kb genomic sequences surrounding the *pannier* locus were extracted from the genome assembly of each allele in *H. axyridis* (*h<sup>C</sup>, *h<sup>A</sup>, *h*) and *C. septempunctata* (*C. sep*). Strain names are given in parentheses. Arrows indicate genes predicted by the exonerate programme (Orange, *pannier*; Blue, GATA transcription factor genes paralogous to *pannier*; Green, other genes). Gene names are listed at the top. Vertical or diagonal bars connecting adjacent genomic structures indicate BLAST<sup>47</sup> hit blocks (bluish, forward hit; reddish, reverse hit). The colour code for colouring the bars is at the bottom. The upper half (1st intron) of the *pannier* locus is diversified (whitish) between different *h* alleles in *H. axyridis*, and shows traces of inversions (crossed reddish bars). Several intronic sequences are conserved between *H. axyridis* and *C. septempunctata* (bars located in the upper half of *pannier* in *C. sep*). The black arrow indicates the region specifically expanded in *H. axyridis*. **b**, Overview of the size of the upper noncoding regions (the 1st intron + the upper intergenic region) at the *pannier* locus in holometabolous insects. The topology of the phylogenetic tree of surveyed insects is adapted from ref. 48 (Coleoptera), and TIMETREE<sup>49</sup> (Diptera, Hymenoptera, Lepidoptera). The sizes of the 1st intron are given in parenthesis if cDNA information was available. *H. axyridis* has the largest noncoding sequence at the *pannier* locus. In some species, synteny of the three paralogous GATA genes was broken up by translocation (*) or insertion (**). **c**, **d**, ML phylogenetic trees constructed with nucleotide sequences of *pannier* coding region (**c**), and those of the conserved region in the 1st intron (**d**). The trees were drawn to scale with branch lengths measured in the number of substitutions per site. Bootstrap values were calculated from 1000 resampling of the alignment data.
b  size comparison of noncoding regions at pnr loci (1st intron + upstream region)

Diptera
- Drosophila melanogaster (fly)
- Anopheles gambiae (mosquito)
- Aedes aegypti (mosquito)

Lepidoptera
- Bombyx mori (moth)
- Mamestra configurata (moth)
- Danausplexippus (butterfly)
- Tribolium castaneum (flour beetle)
- Aethina tumida (small hive beetle)
- Anoplophora glabripennis (long-horned beetle)

Coccinella septempunctata (ladybird beetle)
- Harmonia axyridis (ladybird beetle)
- Onthophagus taurus (dung beetle)
- Nicrophorus vespilloides (burying beetle)
- Aquatica lateralis (firefly)
- Photinus pyralis (firefly)
- Aggrlus planipennis (jewel beetle)
- Pogonius chalcoides (small ground beetle)

Coleoptera
- Apis mellifera (bee)
- Linopteris humilis (ant)
- Nasonia vitripennis (wasp)

Hymenoptera
- C. sep
Moreover, we found that in *H. axyridis*, the size of upstream noncoding sequences of the *pannier* locus (including the 1st intron and the upstream intergenic region) are 46–65 kb larger than the currently available corresponding genomic sequences of the other holometabolous insects (Fig. 4b, *H. axyridis*, 153–172 kb; other holometabolous insects, 13–107 kb). Comparison of the exon-intron structures of *H. axyridis* to those of some of the holometabolous insects also suggested that especially the 1st intron of *pannier* is expanded in *H. axyridis* (*H. axyridis*, 108–118 kb; the other holometabolous insects, 11–44 kb). The expanded region in *H. axyridis* included at least four transcription initiation sites of *pannier* transcripts (Fig. 4c, *pnr-1A–4B*). In addition, in this region, several known DNA-binding motifs of transcription factors involved in *Drosophila* wing formation were more enriched allele-specifically than those in the other genomic regions (Table 1a). For
example, the highly conserved Scalloped (SD) DNA binding motif of the insect wing selector transcription factor complex Vestigial/Scalloped\textsuperscript{24,25} occurred frequently in upstream and downstream regions of the first intron of \textit{pannier} specifically in the $h^C$ allele (Table 1a, the upstream and downstram regions of the 1st intron, $sd$; Supplementary Table 5).
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 |
|--------|-----------------------------|-------------------------------|---------------------------------|
| *hC*   | EcR, foxo                   | Myc, en, *exd*, HLH106, *h*, ss, *pan* | *B-H1*, *ab*, *crc*, *crol*, *Dr*, *rn*, *sd* |
| *hA*   | *exd*, *pan*, *vvI*         | *ab*, *Spps*, *brk*, usp, *pnI* | usp |
| *h*    | *kni*, *rn*, *sqz*          | *ato*, *ab*, *Ets2I*C, *rn*, *sqz* | *al*, *B-H1*, *lns*, *nub*, *sqz*, *tup* |
| C. *sep* | *ato*, EcR, *h*, ss, *tgo* | *B-H2*, *eg*, *tai*, *pnI*, *tgo* | *E(spl)mb*, *ken*, *brk*, *tgo*, *h*, *tai* |

**b. Commonly enriched motifs**

| group | upstream intergenic region | upstream region of 1st intron | downstream region of 1st intron |
|-------|-----------------------------|-------------------------------|---------------------------------|
| Harmonia | Mad, *brk*, *ab*, *taxi*, *tgo* | *lns*, *exd*, *hhk*, *tgo*, *tai*, Mad | *Dr*, *en*, *inv*, *Med*, *sens*, *slou*, *unpg* |
| Harmonia & Coccinella | Mad | *lns*, *exd*, *hhk*, *tgo*, *tai*, Mad | — |

**c. Region size (bp)**

| strain | upstream intergenic region | upstream region of 1st intron | downstream region of 1st intron |
|--------|-----------------------------|-------------------------------|---------------------------------|
| *hC*   | 38,192 (F2-3) / 51,566 (NT6) | 71,107 (F2-3) / 76,264 (NT6) | 47,450 (F2-3) / 44,236 (NT6) |
| *hA*   | 42,826                       | 75,417                        | 52,210                          |
| *h*    | 40,778                       | 67,340                        | 67,340                          |
| C. *sep* | 20,370                      | 56,564                        | 18,432                          |
Furthermore, the RNA-seq data for the $h^c$ background also revealed that the $sd$ co-activator gene *vestigial* was the only transcription factor gene that was significantly upregulated in the future black region from early pupal stages (Supplementary Figure 7), implicating Vestigial as one of the upstream trans-regulatory factors acting together with Sd to form the two-spotted elytral colour pattern of $h^c$. It is noteworthy that the non-coding region of *pannier* in each allele possesses putative DNA-binding motifs that can respond to variety of developmental contexts such as anterior-posterior patterning$^{26-28}$ (*en, inv*), wing fate specification$^{17,24,29}$ (*sd*), hinge-wing blade patterning$^{17,18,30,31}$ & wing vein patterning$^{29,32-34}$ (*nub, rn, ab, al, B-H1, B-H2, ss, hth, exd, kni, brk, vvl, Mad, Med, h*), hormonal cues$^{35,36}$ (*EcR, usp, tai*), and auto-regulation (*pnr*) (Table 1a). These results suggest that allele-specific
elytral colour patterns of *H. axyridis* may be formed by integrating appropriate combinations of developmental contexts of wing formation shared among insects.

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

pannier locus, we performed *de novo* assembly of the *C. septempunctata* genome using a

linked-read sequencing platform (10x Genomics Chromium system), and obtained a

contiguous genomic scaffold including the panner locus (2.41 Mb, Supplementary Figure 4d, Supplementary Table 3). Whereas the noncoding sequences of *C. septempunctata* pannier

are enriched with several species-specific DNA binding motifs (Table 1a, *C. sep*), we found

DNA-binding motifs commonly enriched between *Harmonia* and *Coccinella*, which are

associated with wing vein formation and wing/body wall patterning (*Mad, hth and exd)*\(^{29,31,32}\) (Table 1b). Therefore, co-option of such wing developmental modules in the regulatory

region may have facilitated acquisition of a novel expression domain of *pannier* in pupal

elytral blades in ladybird beetles.
In order to explore the history of the emergence of elytral colour patterns in *H.*

*axyridis*, we also performed a molecular phylogenetic analysis focusing on the highly

conserved *pannier* intronic sequences shared among *H. axyridis* alleles and *C.*

*septempunctata* (3 blocks, totalling 1.1 kb in length, Supplementary Data 1). The maximum

likelihood (ML) phylogenetic tree inferred from nucleotide sequences of the *pannier* coding

region did not resolve the phylogenetic relationship among the alleles in *H. axyridis* to a

satisfactory level (Fig. 5d, bootstrap values < 75). However, the ML tree inferred from the

conserved intronic sequence suggested that in *H. axyridis* the contrasting colour patterns of

the *h* allele (black spots in red background) and the other three alleles (red spots in black

background) diverged first. The latter three alleles diverged more recently (Fig. 5e, bootstrap

values > 90).
Discussion

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

The common ancestor of *Harmonia* and *Coccinella* (Coccinellinae) diverged more than 33.9 million years ago, according to molecular phylogenetic analyses and fossil records\(^37,38\). Therefore, the elytral colour-patterning function of *pannier* shared between *H. axyridis* and *C. septempunctata* was most likely acquired before this divergence event. The
1.1 kb sequence blocks in the 1st intron of *pannier* conserved between *H. axyridis* and *C. septempunctata* are a likely candidate for a regulatory element associated with the ladybird beetle-specific elytral expression of *pannier* in the pupal elytra. The effects of enhancer activities of these sequence blocks have not yet been experimentally addressed. However, the acquisition of such regulatory sequences during evolution would have coincided with the acquisition of the elytral colour patterning function of *pannier* (Fig. 6, blue diamond). These conserved sequence blocks are located in the expanded intronic region specific to *H. axyridis* (Fig. 5a, black arrow). Therefore, the expansion of the 1st intron in the ancestral lineage of *H. axyridis* (Fig. 6, intronic expansion) might be one of the events that facilitated diversification of the intraspecific elytral colour patterns.
Figure 6. Evolutionary model for the acquisition of the highly diversified intraspecific elytral colour patterns of *H. axyridis*. See details in the Discussion.
In the genus *Harmonia*, colour patterns similar to those encoded by the *h* allele and those of *C. septempunctata* (black spots in red background) are commonly observed. Also, the position of the spots is similar across species (e.g. *H. quadripunctata*, *H. octomaculata*, and *H. dimidiata*). Therefore, we speculate that the intronic sequence of *pannier* in the *h* allele of *H. axyridis* might retain a repertoire of regulatory sequences acquired in a common ancestor of the genus *Harmonia* (Fig 6, green arrowhead). However, in the ancestral lineage of *H. axyridis*, the regulatory region of *pannier* appears to have been modified to generate novel colour patterns of the recently diverged alleles (*h*\(^C\), *h*\(^Sp\) and *h*\(^A\); red spots in black background; Fig. 6 magenta, red and purple arrowheads). The 46–65 kb-scale intronic region at the *pannier* locus that is specifically expanded in *H. axyridis* (Fig. 6, intronic expansion, yellow box) might have facilitated accommodation of the allele-specific regulatory motifs.
responsible for the diversified colour pattern of elytra. In addition, traces of inversions in this
region consistently found in allele comparisons suggest that repeated inversions in this region
(Fig. 6, white arrowheads) created opportunities to diverge the noncoding sequence of
*pannier* to successively generate novel diverse alleles within a species by suppressing
recombination within this region. Such inversion events would have occurred in the common
ancestor of *H. axyridis* and its reproductively isolated sister species, *H. yedoensis* because the
major elytral colour patterns are shared between the two species. Large-scale chromosomal
inversion is believed to be one of the major driving forces generating and maintaining
intraspecific morphological variation within a species. Our study exemplifies that not
only a single inversion event but also repeated inversion events at an expanded intron can
lead to the acquisition of novel morphological traits within a species.
From the viewpoint of evolutionary developmental biology it is noteworthy that in

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

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

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

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

regulate multiple intraspecific wing colour patterns prevailing in the population\(^4^4\). This

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

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

diverged from ancestral species of Cucuoidea\(^3^7\) (Fig. 5a, Cucuoidea), leaf-litter or

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

colourful and more simply patterned forewings (elytra) than the ancestors of butterflies,
Therefore, these ancestors presumably would have possessed far fewer colour pattern regulatory genes. In *H. axyridis*, this developmental constraint may have led to the selection of *pannier* as the major evolvable gene to a signal-integrating ‘input-output’ regulatory gene\(^\text{45,46}\). This might have generated more than 200 colour patterns genetically tightly linked to the *h* locus by utilising the expanded regulatory DNA sequence. Future research aiming to identify specific regulatory inputs to *pannier* will help clarify the regulatory mechanisms underlying the generation of highly diverse intraspecific polymorphism at the interspecific level. Another important issue to clarify whether *pannier* is indeed the hotspot of morphological evolution in ladybird beetles is whether *pannier* is responsible for the remaining >20 minor colour patterns in *H. axyridis*. 
Methods

Insects

Laboratory stocks of *H. axyridis* and *C. septempunctata* were derived from field collections in Japan. They were reared at 25°C and usually fed on artificial diet, or fed on the pea aphid *Acyrthosiphonpisum* (kindly provided by Dr. T. Miura) for egg collection.

Larvae and pupae analysed in this study were not sexed.

Phenoloxidase (PO) activity staining

Pupa elytral discs were dissected in a potassium phosphate buffer (100 mM KH$_2$PO$_4$/K$_2$PO$_4$, 150 mM NaCl, pH 6.3) on ice. PO staining was performed using 0.4 mg/ml dopamine as a substrate for 2 hours at room temperature as previously described. After
washing several times in the potassium phosphate buffer containing 0.3% Triton-X100 and mounted in this solution. Images were captured with a stereoscopic microscope (MZ FLIII, Leica) equipped with a digital camera (DP70, Olympus).

**Histological analysis**

To visualise tissue morphology and PO active tissues, pharate adult elytra dissected in ice-cold PBS (137 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, pH 7.2) at 96 h AP or those after PO activity staining were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min on ice and for 75 min at room temperature. After fixation, the elytra were washed several times in 100% methanol and stored in 100% methanol at −20°C until use. After dehydration, the elytra were embedded in 4% carboxymethyl cellulose (CMC; FINETEC), and were
frozen in hexane cooled with dry ice. The freeze-embedded elytra were stored at −80°C until use. The 6 μm frozen sections were prepared as described previously using an adhesive film (Cryofilm Type 1; FINETEC). Sections of the PO activity stained elytra were mounted in PBS and photographed using IX70. For nuclear and F-actin staining, sections were treated with 2.5 μg/ml propidium iodide, 1 mg/ml RNase A and 5 U/ml AlexaFluor 488 phalloidin (Molecular Probes) for 1 hour at 37°C under a dark condition. After washing three times in PBS, the sections were mounted in an antifade reagent (FluoroGuard™; Bio-Rad), and images were captured with a confocal laser-scanning microscope (LSM 510; Carl Zeiss).

For localisation of carotenoid, elytra at 96 h AP were embedded and sectioned as described above. All procedures were rapidly performed to prevent diffusion of carotenoids.

The sections were dried for 1 min, mounted in PBS and immediately photographed using
Larval and pupal elytral discs and pharate adult elytra of *H. axyridis* (*h*) and *C. septempunctata* were dissected in PBS on ice. Soon after dissection, the tissues were frozen in liquid nitrogen and stored at –80°C until use. Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen) or RNeasy Micro Kit (Qiagen) according to the manufacture’s instructions, and treated with 2 U DNase I (Ambion) for 30 min at 37 °C. The first-strand cDNA was synthesised with SMARTer PCR cDNA Amplification Kit (Clontech) using 1 μg of total RNA according to the manufacture’s instructions. *H. axyridis* and *C. septempunctata* cDNA fragments were amplified by reverse transcription-polymerase chain
reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) with the primers listed in Supplementary Table 6–8. The PCR product was cloned into the EcoR V site of the pBluescript KS+ vector (Stratagene) or pCR4-TOPO vector (TOPO TA Cloning Kit; Invitrogen). The nucleotide sequences of the PCR products were determined using a DNA sequencer 3130 genetic analyser (Applied Biosystems). The SNPs in open reading frame (ORF) of pannier were determined through direct sequencing of the PCR products treated with ExoSAP-IT (Affymetrix). Sequencing was performed by DNA sequencing service (FASMAC) using the primers listed in Supplementary Table 6. Sequence analysis was carried out using DNASIS (Hitachi Software Engineering) or ApE $^{52}$ (version 2.0.45) software. Nucleotide sequences and deduced amino acid sequences were aligned with
ClustalW in MEGA$^{53}$ software (version 7.0.18) and the alignment figure was generated using Boxshade$^{54}$ (version 3.21).

**Gene expression analysis by RT-PCR**

For the gene expression analysis in each developmental stage, elytral tissues of three individuals of *H. axyridis* ($h^C$) and *C. septempunctata* were dissected as described above. Six elytral tissues from each sampling stage were pooled in one tube. Total RNA extractions and the subsequent first-strand cDNA syntheses (using 425 ng and 267 ng of total RNA for *H. axyridis* and *C. septempunctata* samples, respectively) were performed as described above. Three $\mu$l of 100 and 62.8 times diluted *H. axyridis* and *C. septempunctata* first-strand cDNA was used as a template for PCR, respectively. The PCR cycle numbers are
35 for all genes. A set of primers #1 and #2 for each gene was used for this analysis (Supplementary Table 7).

For the gene expression analysis in the future red and black regions, red and black regions of pharate adult elytra at 84 h AP were bored with a needle. Internal diameters of 0.7 and 0.6 mm were used for *H. axyridis* and *C. septempunctata*, respectively. In the case of *C. septempunctata*, elytra stained with PO activity were used for boring since carotenoid localisation was not observable unlike *H. axyridis*. cDNA synthesis was performed as described above, using as much total RNA as we could extract. 20 μl of 10 times diluted first-strand cDNA was used as a template for PCR. The PCR cycle numbers are 45 cycles for *Ha-pnr*, 38 cycles for *Ha-rp49*, 47 cycles for *Cs-pnr* and 40 cycles for *Cs-rp49*. A set of primers #3 and #4 for each gene was used for this analysis other than *rp49*. *Ha-rp49* and
Cs-rp49 were used as internal controls. Reactions without reverse transcriptase were performed with cDNA synthesis as negative control samples for the RT-PCR experiments. No band was detected in these reactions for all genes. The primers used for this analysis are described in Supplementary Table 7.

**Larval RNAi**

dsRNA synthesis and microinjection into larvae were performed as previously described. The cloned cDNA fragments amplified by PCR were used as templates for dsRNA synthesis. Approximately 1.4 to 2.7 μg and 1.4 to 2.0 μg of the dsRNAs of Ha-pnr and Cs-pnr were injected into two-day-old forth (final) instar larvae, respectively. Approximately 2.0 to 2.7 μg and 1.4 to 2.7 μg of the EGFP dsRNA were injected into H.
axyridis and C. septempunctata larvae as negative controls, respectively. Different amount of dsRNA for each gene in this range gave no difference in phenotypic effects (data not shown).

In order to give enough time for the completion of pigmentation, images of adults were captured at more than 2 days after eclosion using a digital microscope (VHX-900, Keyence).

**In situ hybridisation**

Essentially the same protocol for whole mount pupal antennal primordia of the silk moth was used. To increase RNA probe penetrance in elytral epidermis covered with sclerotised cuticle at 76–84 h AP, the peripheral edge of an elytron was cut off, and then, ventral and dorsal elytral epidermis layers appressed together were carefully separated with fine forceps after fixation. To reduce non-specific probe hybridisation, fixed, separated and
detergent-permeabilised elytra epidermal samples were stored in 100% methanol for more than 12 hours at −30°C, and prehybridisation treatment was extended to two overnights. The ventral epidermis samples were not used for analysis because of high non-specific background signals. pannier antisense probes were designed at 5' and 3' regions of ORF excluding the two conserved GATA zinc finger coding regions in the middle to prevent cross-hybridisation with other GATA family genes. The PCR primers used to amplify the template DNA for in vitro RNA probe synthesis were listed in Supplementary Table 9. Briefly, pannier ORF fragment was amplified by RT-PCR using cDNA from 72 h AP (h^C), and cloned into pCR4-TOPO vector (Invitrogen). Sense and antisense probe templates were amplified from the cloned cDNA. Sense and antisense riboprobes were transcribed using the flanking T7, T3 or SP6 promoter sequences. Mixture of 5' and 3' probes was used for hybridisation.
De novo genome assembly of *H. axyridis*

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


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

Adaptor sequences and low-quality regions in paired-end and mate-pair reads were trimmed using Platanus_trim (version 1.0.7) with default parameters. Trimmed reads were assembled by Platanus (version 2.0.0), which was derived from Platanus to assemble haplotype sequences (i.e. haplotype phasing) instead of consensus sequences. Procedures of Platanus2 are briefly described as follows:

(1) De Bruijn graphs and scaffold graphs are constructed without removal of bubble structures caused from heterozygosity. Paths that do not contain junctions correspond to assembly results (scaffolds). Scaffold pairs in bubbles represent heterozygous haplotypes.
(2) Paired-ends or mate-pairs are mapped to the graphs to detect links between bubbles, and
linked bubbles are fused to extend haplotype sequences.

(3) Each haplotype (contig or scaffold) is independently extended by modules of \textit{de novo}
assembly derived from Platanus.

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

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

(6) Homologous pairs of scaffolds are formatted into bubble structures as output. For each
pair, longer and shorter scaffold were called "primary-bubble" and "secondary-bubble",
respectively. Primary-bubbles, secondary-bubbles and non-bubble scaffolds are collectively
called "phased-scaffolds".
In addition, Platanus2 can connect primary-bubbles and non-bubble scaffolds to construct long "consensus scaffolds", which consists of mosaic structure of haplotypes (i.e. paternal and maternal haplotypes are mixed). Employing the strategy of Platanus2, certain highly heterozygous regions were expected to be assembled contiguously compared to Platanus. Using the markers of the responsible region of wing pattern (pannier-locus), we found that two long bubbles and one short non-bubble scaffold corresponded to the locus. Consequently, one consensus scaffold was constructed from these phased scaffolds, which covering the breakpoint markers at the pannier locus. We used that consensus scaffold (3.13 Mb) for the downstream in silico sequence analyses. We assessed the completeness of the genome assemblies using BUSCO\textsuperscript{60} (version 3.0.2, Insecta dataset [1,658 orthologs]).
DNA sequencing for *de novo* genome assembly by long read and linked-read platforms

High molecular weight (HMW) genomic DNA was extracted using QIAGEN Genomic-tip 100/G (QIAGEN) according to the manufacturer’s instructions. The concentrations and qualities of the extracted HMW genomic DNA were evaluated using Qubit dsDNA, and RNA HS kits (Thermo Fisher).

For library preparation for 10x Genomics Chromium system, one pupa (*h*\(^C\) [NT6 strain] and *h* [NT8 strain]) or one adult (*h*\(^A\) [F2 adult progenies in genetic cross *h*\(^A\) × *h*\(^Sp\)] and *C. septempunctata* [MD-4 strain]) was used. Size selection by BluePippin (range: 50kb–80kb, Sage Science) was performed only for *h*\(^A\) genomic DNA used in 10x linked-read library preparation.
Preparation of Gel Bead-in-Emulsions (GEMs) for each 10x Genomics Chromium library was performed using 0.5–0.6 ng of HMW genomic DNA according to the manufacturer’s instructions. The prepared GEMs were quality checked using Qubit dsDNA HS kit (Thermo Fisher) and Bioanalyzer (Agilent), and processed with Chromium Controller (10x Genomics). The constructed DNA libraries were quality checked again in the same way.

Sequencing of the libraries was performed in the Hiseq X ten (Illumina) platform (1 library/lane) at Macrogen. In total, we generated 66.9 Gb, 64.6Gb, 64.9 Gb and 60.3 Gb of raw reads for linked-read sequencing (\(h^C\), \(h^A\), \(h\), and \(C. septempunctata\), respectively).

For library preparation for PacBio system, ten to eleven pupae (\(h^C\) [NT6 strain] and \(h\) [NT8 strain]) or adults (\(h^A\) [F2 adult progenies in genetic cross \(h^A \times h^{sp}\)]) were used.
The libraries were prepared according to the 20-kb Template Preparation Using BluePippin™ Size-Selection System (Sage Science, MA, USA). In total, 4.31 Gb, 4.92 Gb and 4.44 Gb of insert sequences (approximately 10 × coverage of the genome, assuming a genome size of 423 Mb) were obtained from 4 to 5 SMRT cells for $h^A$, $h^C$ and $h$, respectively.

De novo assembly of 10x linked-reads

For 10x linked-reads libraries of four samples (three $H. axyridis$ and one $C. septempunctata$), Supernova (version 2.0.0) was executed with default parameters except for the maximum number of used reads (the --maxreads option) to obtain the optimum coverage depth for Supernova (56×). For each sample, the value for --maxreads was determined as follows:
(1) Barcode sequences in raw linked-reads were excluded using "longranger basic" command of Long Ranger\textsuperscript{62} (version 2.1.2), resulting in "barcoded.fastq" file.

(2) Adaptor sequences and low-quality regions in "barcoded.fastq" were trimmed using Platanus\textunderscore trim (version 1.0.7) with default parameters.

(3) 32-mers in the trimmed reads were counted by Jellyfish\textsuperscript{63} (version 2.2.3) using the following two commands and options:

```bash
$ jellyfish count -m 32 -s 20M -C -o out.jf barcoded_1.trimmed barcoded_2.trimmed

$ jellyfish histo -h 1000000000 -o out.histo out.jf
```

In summary, all 32-mers in both strands (-C) were counted and distribution of the number of occurrences without upper limit of occurrences (-h 1000000000).
(4) The haploid genome size was estimated using the custom Perl script. For the distribution of the number of 32-mer occurrences ("out.histo"), the number of occurrences corresponding to a homozygous peak was detected, and the total number of 32-mers was divided by the homozygous-peak-occurrences. Here, 32-mers whose occurrences were small (< the number of occurrences corresponding to the bottom between zero and heterozygous peak) were excluded for the calculation to avoid the effect from sequencing errors.

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

\[
\frac{\text{estimated-haploid-genome-size}}{\text{mean-read-length-of-barcoded.fastq}} \times 56
\]

As a result, we obtained the scaffolds including the genes surrounding \(H.axyridis-pannier\) (\(h^C\) [NT6], 2.74 Mb; \(h^A\) [F2 hybrid], 1.42 + 1.61 Mb; \(h^C\) [NT8] 2.79 Mb), and homologous regions in \(C. septempunctata\) (haplotype 1, 10.16 + 2.41 Mb; haplotype 2,
10.13 + 2.44 Mb). We used those sequences for the downstream *in silico* analyses. We assessed the completeness of the genome assembly using BUSCO\textsuperscript{62} (version 3.0.2, Insecta dataset [1,658 orthologs]).

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

Concerning the genome assemblies of *H. axyridis*, we used minimap2\textsuperscript{64} (ver. 2.9) and PBjelly\textsuperscript{65} (ver. PBSuite_15.8.24) software to fill gaps around the *pannier* locus. In each genome of three strains of *H. axyridis*, we first mapped PacBio reads to the assembly generated from the 10x linked-reads using minimap2. Then, we chose PacBio reads mapped to the scaffold containing *pannier* gene. These PacBio reads were subjected to gap-filling of
the scaffold with PBjelly. We obtained gap-free nucleotide sequences spanning the entire

pannier locus and the upstream intergenic regions.

Concerning the genome assembly of *C. septempunctata*, there was a single gap estimated to be 15kb long by Supernova programme in the 1st intron of *pannier* locus. We handled this gap region as repeated N, and included it in the downstream *in silico* analyses.

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

For the *H. axyridis* F2-3 sample, trimmed reads of the 15 kbp-mate-pair library were mapped to the consensus scaffold set of Platanus2 using BWA-MEM\(^6\) (version 0.7.12-r1039) with default parameters. Next, a consensus scaffold corresponding to *pannier*-locus was
segmented into 2 kbp-windows, and links between windows (the number of mate-pairs ≥ 3) were visualised by Circos\textsuperscript{67} (version 0.69-6).

Additional comment for the editor and reviewers.

Although Platanus2 is not open currently, we plan to release the same version (2.0.0) of Platanus2 at the web site of Platanus (http://platanus.bio.titech.ac.jp/) by the publication of this study.

Resequencing of *Harmonia* genome for choosing appropriate strains for Genome Wide Association Study (GWAS)
Genomic DNA was extracted from each of $h^C$ (F6 strain), $h^A$ (NT3 strain) and $h^{Sp}$ (CB-5 strain), and used to create Illumina libraries using TruSeq Nano DNA Sample Preparation Kit (Illumina) with insert size of approximately 400 bp. These libraries were sequenced on the Illumina HiSeq 1500 using a 2 x 106-nt paired-end sequencing protocol, yielding 84.7M paired-end reads. SNP site identification was conducted basically according to GATK Best Practice (ver. August 7 2015). After trimming adaptor sequences with Cutadapt software (ver. 1.9.1), the sequence data were mapped to the de novo genome assembly data using bwa software (ver.0.7.15, BWA-MEM algorithm). Sequences and alignments with low quality were filtered using Picard tools (ver. 2.7.1) and GATK software (ver. 3.6 and 3.7), and 73,4443 SNP markers in the strains were identified. The most
distantly related strains (\(h^c\) [F6 strain] and \(h^A\) [NT3 strain]) were selected by performing phylogenetic analysis using SNPhylo\(^7\) (Version: 20140701).

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

For each pair of scaffolds, we constructed dot plots by performing pairwise-alignment using "nucmer" programme in the MUMmer package\(^7\) (version 3.1).

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

(2) *H. axyridis* vs. *C. septempunctata*; `-l 8 -c 20`. Alignment results (delta file) were input into "mummerplot" programme to generate dot plots. Note that resultant gnuplot scripts resulted from mummerplot were edited for visualization.
We also visualised the homology and structural differences between the 700 kb-genomic region including *pannier* using Easyfig\textsuperscript{73} (ver. 2.2.2). Short BLAST\textsuperscript{47} hit fragments less than 500 bp, and putative short repeat sequences less than 1250 bp, which showed more than two BLAST hit blocks within the 700 kb region, were filtered using a custom Perl script. Exon-intron structures of putative genes in the 700 kb regions were obtained using Exonerate\textsuperscript{74} (ver. 2.2.0) with the options `-m est2genome --showvulgar yes --ryo ">%qi length=%ql alnlen=%qa\n>%ti length=%tl alnlen=%ta\n" --showtargetgff yes --showalignment no --score 2000`. cDNA sequences cloned by RT-PCR or predicted by RNA-seq were used as queries. If a single cDNA unit was split into multiple fragments, we merged the fragments by performing exonerate search again using the cDNA sequences whose subsequences were substituted by the genomic hit fragments in the first exonerate
search as a query. Exonerate output files were converted to the GFF3 format using our

bug-fixed version of the process_exonerate_gff3.pl with the option ‘–t EST’. The GFF3 file

and a FASTA format file of each scaffold were converted to a GENBANK format file using

EMBOSS Seqret programme (ver. 6.6.0.0) with the options ‘-fformat gff -osformat genbank

’. The GENBANK format files corresponding to the 700 kb genomic sequences surrounding

pannier, which were used as input files of Easyfig, were extracted using the

Genbank_slice.py Python script (ver. 1.1.0).

Flexible ddRAD-seq

We newly constructed a flexible ddRAD-seq library preparation protocol to

facilitate high-throughput ddRAD-seq analyses at low cost. We designed all enzymatic
reactions to be completed sequentially without DNA purification in each step to make the procedures simple. In addition, we designed 96 sets of indexed and forked sequencing adaptors compatible with Illumina platform sequencers (Supplementary Table 10).

Briefly, 100 ng of genomic DNA was first double-digested with 15 U of EcoRI-HF and 15 U of HindIII-HF in 20 µl of NEB CutSmart Buffer (New England Biolabs) at 37°C for 2 hours. 15 µl of the digested DNA, 4 pmoles of adaptor DNA, 10 µmole of ATP, 400 Units of T4 DNA ligase were mixed in 20 µl, incubated at 22 °C for 2 hours, and denatured at 65°C for 10 minutes. Ligated library DNA fragments were purified with Agencourt AMPureXP (Beckman Coulter) according to the manufacturer’s instructions. Library DNA fragments ranging from 300 bp to 500 bp were size-selected with Pippin Prep (Sage Science).

Concentration of each library DNA was quantified using KAPA Library Quantification Kits.
(Roche) according to the manufacturer’s instructions. Sequence data were obtained by applying 96 DNA libraries to a single lane of Hiseq 1500 (Illumina).

**Linkage map construction and GWAS**

A single $h^C$ male (F6 strain) and a single virgin $h^A$ female (NT3 strain) were crossed, and the obtained F1 progenies were backcrossed with the F0 male ($h^C$, F6 strain). Finally, 183 adult F2 progenies ($h^C = 80$, $h^C/h^A = 103$) and 2 F0 adults were collected for RAD-sequence analysis, and stored at $-30^\circ C$ until use. Genomic DNAs were extracted individually using an automatic nucleic acid extractor (PI-50α, KURABO). Briefly, each frozen ladybird beetle and a zirconia bead were transferred to 2 ml plastic tube (Eppendorf) on ice. Immediately, 250 µl of cold lysis buffer including Proteinase K and RNase, but not SDS was added to the
sample, and the tubes were vigorously shaken with a tissue grinder (Tissue Lyser LT, Qiagen) at 3000 rpm for 1 min. Then, 250 µl of lysis buffer including SDS was added to each crushed sample, and processed with the programme for DNA extraction from mouse tail, according to the manufacturer’s instructions. The extracted genomic DNA was diluted in 30 µl of TE buffer. The DNA concentration of each sample was quantified using Qubit dsDNA BR Kit according to the manufacturer’s instructions (Thermo Fisher Scientific). We performed flexible ddRAD-seq using these genomic DNA samples. 0.6–6.0 millions (mean = 2.0 millions) of 106 bp paired-end reads per sample were generated using two lanes of Hiseq 1500 (Illumina) following the methods in the User Guide.

Mapping and polymorphic site calling were conducted as described in the resequencing analysis above except that the procedure for filtering duplicated reads using
Picard was eliminated because we did not amplified DNA library by PCR. Count data at each SNP sites were extracted from the obtained vcf file using vcf_to_rqtl.py script in rtd software with the options '5.0 80'. To avoid programme errors, we modified the script to skip the read depth data (DP) including characters in the GATK vcf file.

We constructed a linkage map using R/qtl (version 1.42.8) and R/ASMap packages according to the QTL mapping workflow for BC1 population of Jaltomata. Using the obtained csv file as an input, we eliminated the polymorph sites that behaved as located on the X chromosome. In addition, individuals with low mapping quality, and marker sites with low quality or highly distorted segregation patterns were eliminated as well. Finally, 4419 markers sites, and 177 F2 individuals were used. The linkage map was initially constructed with mstmap programme (R/ASMap) with the options 'dist.fun =
"kosambi", p.value = 1e-25', and highly linked linkage groups were merged manually. The markers consistently incongruent with neighboring markers were eliminated using correctGenotypes.py\textsuperscript{82} script with the options '-i csvr -q 0.1 -t 4.0'.

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

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

In addition to the genetic cross in GWAS ($h^C \times h^A$), two independent crosses ($[h \times h^C]$ and $[h^A \times h^{Sp}]$) were performed.
In the former cross, a single $h$ male (D-5 strain) and a single virgin $h^C$ female (F2-3-B strain) were crossed, and the obtained F1 progenies were sibcrossed. Finally, 80 F2 adult progenies ($h^C = 30, h^C/h = 34$, and $h = 16$) were collected for genotyping, and stored at $-30^\circ$C until use. In the latter cross, a single $h^{Sp}$ male (CB-5 strain) and a single virgin $h^A$ female (NT3 strain) were crossed, and the obtained F1 progenies were sibcrossed. Finally, 273 F2 adult progenies ($h^{Sp} = 103, h^{Sp}/h^A = 80$, and $h^A = 90$) were collected for genotyping, and stored at $-30^\circ$C until use.

Genomic DNA was extracted individually using the automatic nucleic acid extractor (PI-50α, KURABO) as described in the previous section, and diluted to approximately 100 ng/μl. We searched for genotyping markers by amplifying and sequencing the intronic region of the genes surrounding pannier with PCR. The individual
PCR was performed using approximately 100 ng of genomic DNA and Q5 DNA polymerase (New England Biolabs) with 45 cycles. The primers used, the markers identified and the typing results are summarised in Supplementary Table 2.

RNA-seq analysis

The total RNA extraction procedure for RNA-seq is essentially the same as that for the gene expression analysis in the presumptive red and black regions by RT-PCR. The same strain used for de novo genome sequencing (F2-5 strain, $h^C$) was used. In total, 12 samples (2 colours [Black/Red] $\times$ 2 developmental stages [24h AP/72h AP] $\times$ 3 biological replicates) were prepared for RNA-seq analysis. Two fragments of bored epidermis from left and right elytra were collected as a single sample in each condition. All total RNA extracted from each
sample (12 ng to 158 ng) using RNeasy Mini Kit (QIAGEN) and QIAcube (QIAGEN) was
used for each cDNA library preparation. RNA-seq library preparation was performed using
the SureSelect strand specific RNA library prep kit (Agilent) according to the manufacturer’s
instructions. Briefly, mRNA was purified using Oligo-dT Microparticles. The strand-specific
RNA-seq libraries were prepared using dUTP and Uracil-DNA-Glycosylase. The libraries
and its intermediates were purified and size-fractionated by AMPure XP (Beckman Coulter).
For quality check and quantification of the RNA-seq libraries, we employed 2100
Bioanalyser and DNA 7500 kit (Agilent). 100 bp paired end read RNA-seq tags were
generated using the Hiseq 2500 (Illumina) following the methods in the User Guide.
In advance of reference mapping, adaptor and poly-A sequences were trimmed
from raw RNA-seq reads by using Cutadapt (ver. 1.9.1)\textsuperscript{69}. Low-quality reads were also
filtered out by a custom Perl script as described previously\textsuperscript{83}. The preprocessed RNA-seq reads were mapped to the reference \textit{H. axyridis} genome sequences using TopHat2\textsuperscript{84} (ver. 2.1.0) with default parameters, and assembled by Cufflinks\textsuperscript{85} (ver. 2.2.1) with the -u option in each sample. All predicted transcript units and all loci from different samples were merged by Cuffmerge in the Cufflinks suite. The RNA-seq read pairs (fragments) mapped to each predicted transcript unit and locus were counted using HTSeq\textsuperscript{86} (ver. 0.6.1) with the options '-s no -t exon -i transcript' and '-s no -t exon -i locus', respectively. The downstream statistic analyses were performed using edgeR\textsuperscript{87,88} package (ver. 3.16.5). The raw RNA-seq fragment counts were normalised by the trimmed mean of M-values (TMM) method. Fold change between black and red regions in each stage and its statistical significance (FDR) were
calculated. The mean fold changes of the genes in the scaffolds including the \textit{h} locus candidate region were visualised with IGV\textsuperscript{89, 90} software (ver. 2.3.88).

Comparison of the sizes of the upper noncoding regions of \textit{pannier} among holometabolous insects

The holometabolous insects, whose genomic sequences are well assembled at the \textit{pannier} locus to the extent that at least the two paralogous GATA transcription factor genes, \textit{GATAe} and \textit{serpent}, are included in the same scaffold, were selected for comparison.

Concerning Coleoptera, genomic sequences were collected from the Genome database at NCBI\textsuperscript{91} (GCA\_000002335.3, Tcas5.2; GCA\_001937115.1, Atum\_1.0; GCA\_000390285.2, Agla\_2.0; GCA\_000648695.2, Otau\_2.0; GCA\_001412225.1,
Nicve_v1.0; GCF_000699045.1, Apla_1.0; GCA_002278615.1, Pchal_1.0) and

Fireflybase\textsuperscript{92} (\textit{Photinus pyralis} genome 1.3, \textit{Aquatica lateralis} genome 1.3). Concerning holometabolous insect other than Coleoptera, genomic information at Hymenoptera Genome Database\textsuperscript{93} (Hymenoptera) (GCF_000002195.4, Amel_4.5; GCF_000217595.1, Lhum_UMD_V04; GCF_000002325.3, Nvit_2.1), Lepbase\textsuperscript{94} (Lepidoptera, butterfly; Danaus_plexippus_v3_scaffolds), SilkBase\textsuperscript{95} (Lepidoptera, silk moth; Genome assembly [Jan.2017]), Flybase\textsuperscript{96} (Diptera, \textit{Drosophila}, dmel_r6.12_FB2016_04), and the Genome database at NCBI\textsuperscript{91} (Diptera, mosquitos; GCA_000005575.1, AgamP3; GCA_002204515.1, AaegL5.0) (Lepidoptera, moth; GCA_002192655.1, ASM219265v1) were utilised. We performed BLAST\textsuperscript{47} search (TBLASTN, ver. 2.2.26) using the amino acid sequence of \textit{H. axyridis} Pannier as a query, and identified \textit{pannier} orthologs by focusing on the top hits, and
the conserved synteny of the three paralogous GATA transcription factor genes, in which

*serpent*, *GATAe*, and *pannier* are tandemly aligned in this order from the 5’ to 3’ direction.

The sizes of the upper noncoding region of *pannier* were estimated by calculating the
difference between the coordinates of the 3’ end of BLAST hit region of *GATAe* and the 5’ hit
region of *pannier*. Traces of translocation or insertions between the paralogous GATA genes
were surveyed by looking into the annotations between the *GATAe* and *pannier* loci. If a
noncoding exon 1 was annotated at the pannier locus, the size of the first intron was also
calculated as well.

Motif enrichment analysis of the upper noncoding regions of *pannier*
To search for the *Drosophila* known DNA-binding sites at *pannier*, 1,139 DNA motifs were retrieved from the JASPAR^{97} database using the MotifDb^{98} R package.

Concerning the SD-binding motif, the position weight matrix (PWM) scores were calculated using the 2,557 ChIP-seq peaks in *Drosophila* genome obtained by Ikmi et al.^{99} and the RSAT peak-motifs programme^{100}. The nucleotide sequences at the three upper noncoding regions of *pannier* (the upper intergenic region, the upstream half of the 1st intron, and the downstream half of the 1st intron) were collected by forging BSgenome^{101} data packages using our *H. axyridis* and *C. septempunctata* genome sequences, and by retrieving the sequences using coordinate information obtained for annotation in Fig. 5a and the GenomicFeatures^{102} R package. We here defined the upstream half of the 1st intron as the region including all of the traces of inversions or corresponding sequences shared among
different h alleles in *H. axyridis*. GRange objects were generated using the coordinate information obtained for annotation in Fig. 5a. Motif enrichment was quantified using the PWMEnrich\textsuperscript{103} R package. As control background genomic regions for the upper intergenic regions of *pannier*, we used 2 kb promoter sequences of 11,279 genes to which RNA-seq reads were mapped (*H. axyridis* genome assembly version 1), and not located within the 10 kb from the end of the genomic scaffolds. As control background genomic regions for the upstream and downstream regions of the 1st intron of *pannier*, we used 2 kb sequences at the 5' and 3' end of the 1st introns, which are more than 2 kb long, and without gaps (2825 and 2810 sequences, respectively). Since there is no reliable gene annotation data for the *C. septempunctata* genome, we used the *H. axyridis* genomic background sequences. Each motif enrichment score, which is related to average time that transcription factors spend in...
binding to a DNA sequence\textsuperscript{104}, was calculated using default parameter of PMWEnrichment.

\textit{br} and \textit{da} DNA-binding motifs were excluded from the analysis.

\section*{Molecular phylogenetic analysis}

Concerning the conserved regions in the upper half of the 1st intron of \textit{pannier}, nucleotide sequences were collected from the BLAST hits obtained to construct Fig. 5a. The collected BLAST hit sequences were arranged in the same directions using a custom Perl script, and aligned using MAFFT\textsuperscript{105} (ver. 7.222). We concatenated three alignment blocks, and manually excluded GAP sites and seemingly nonhomologous sites in the alignment (Supplementary Data 1). Concerning the coding region, nucleotide sequences of the cloned cDNAs were aligned, and trimmed in the same way (Supplementary Data 1).
The maximum likelihood (ML) phylogenetic trees were constructed using RAxML\textsuperscript{106} (ver. 8.0.0) with the options ‘--maxiterate 1000 --localpair --clustalout’. We determined appropriate models of sequence evolution under the AIC\textsubscript{c}4 criterion using Kakusan\textsuperscript{4107}. 100 replicates of shotgun search for the likelihood ratchet were performed. Nodal support was calculated by bootstrap analyses with 1000 replications.

Data availability

The DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank accession numbers for the sequences reported in this article are LC269047–LC269055 for \textit{Ha-pnr}, LC269056 for \textit{Cs-pnr} and LC269057 for \textit{Cs-rp49}. The
genomic sequencing data, resequencing data and the RNA-seq data were deposited in the DNA Data Bank of Japan (DDBJ) under the accession number DRA002559, X (under curation) & DRA006068, and DRA005777, respectively.
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Supplementary Information

Below are supplementary data related to our analyses. Included are one table on our small-scale RNAi screen, one table on statistics of genome assemblies, five tables on DNA primers designed for amplification and sequencing, and five figure legends for the Supplementary data.
Supplementary Table 1 | The 1st smal-scale RNAi screening of wing/body wall patterning genes

| gene name               | phenotype in elytra         | tested alleles |
|-------------------------|----------------------------|----------------|
| *araucan*               | no phenotype               | *h, hC, hSp, hA* |
| *aristaless*            | no phenotype               | *h*            |
| *apterous*              | wrinkled elytra            | *hB, hB*       |
| *blistered*             | lethal                     | *hB, hB*       |
| *Cubitus interruptus*  | pupal lethal               | *hB*           |
| *Distal-less*           | no phenotype               | *h, hB*        |
| *decapentaplegic*       | smaller elytra             | *h, hB*        |
| *Epidermal growth factor receptor* | lethal                  | *hB, hB*       |
| *pannier*               | transformation of black regions to red regions | *h, hB, hB, hB* |
| *wingless*              | no phenotype               | *h, hB*        |
**Supplementary Table 2 | Markers and Primers for gene association study on the h locus**

| Marker/primer name | Target | Sequence (5’-3’ | polymorphism (coordinate) between h-F0 and hC-F0, between hA-F0 and hSp-F0, and between h-A0 and hC-F0 | Recombinants’ genotype | Cross | The h candidate regions |
|--------------------|--------|-----------------|-------------------------------------------------|------------------------|-------|-------------------------|
| mbl-F              | scaffold898_cov129:657,001-657,297 | AAAATGAGAGTGTAAGAAGA A/G (657,138) | h/h-#27 (A/G heterozygote) | h x h^c | < h < h^c |
| mbl-R              | scaffold898_cov129:1,560,979-1,561,277 | CAAAGCAGAGAAACCTAACA | A/T (1,561,052) | no recombinant | h x h^c | h > |
| DUS-F              | scaffold99874_cov139:62,450-62,532 | GAGTCTCCAAAATTACCAT | 80bp/80_or_57bp | h/hC-#7 (57bp homozygote) | h x h^c | h > |
| DUS-R              | scaffold99874_cov139:62,450-62,532 | GAGTCTCCAAAATTACCAT | 80bp/80_or_57bp | h/hC-#7 (57bp homozygote) | h x h^c | h > |
| pnr-F              | scaffold898_cov129:657,001-657,297 | AAAATGAGAGTGTAAGAAGA A/G (657,138) | h/h-#27 (A/G heterozygote) | h x h^c | < h < h^c |
| pnr-R              | scaffold898_cov129:657,001-657,297 | AAAATGAGAGTGTAAGAAGA A/G (657,138) | h/h-#27 (A/G heterozygote) | h x h^c | < h < h^c |
| mus201-F           | scaffold267_cov168:390,452-390,521 | CAAAGGAGGCCTCGAGAATA | 70bp/100bp | h/c/hc-#14 (70bp/100bp heterozygote) | h x h^c | h > |
| mus201-R           | scaffold267_cov168:390,452-390,521 | CAAAGGAGGCCTCGAGAATA | 70bp/100bp | h/c/hc-#14 (70bp/100bp heterozygote) | h x h^c | h > |
| Mink-F             | scaffold99874_cov139:62,450-62,532 | GAGTCTCCAAAATTACCAT | 80bp/80_or_57bp | h/hC-#7 (57bp homozygote) | h x h^c | h > |
| Mink-R             | scaffold99874_cov139:62,450-62,532 | GAGTCTCCAAAATTACCAT | 80bp/80_or_57bp | h/hC-#7 (57bp homozygote) | h x h^c | h > |
| Fam92_3’-F         | scaffold294:205,919-205,999 | GTCTGCTCATTCTAGYGTATGATGA | 80bp~60bp | hA/hSp-#41 (~60bp homozygote) | h^4 x h^sp | h^4 < h^sp |
| Fam92_3’-R         | scaffold294:205,919-205,999 | GTCTGCTCATTCTAGYGTATGATGA | 80bp~60bp | hA/hSp-#41 (~60bp homozygote) | h^4 x h^sp | h^4 < h^sp |
| sens-F             | scaffold267:650,101-650,598 | AGTTGCGTCAGCAATTG | 242bp~250bp | no recombinant | h x h^c | h > |
| sens-R             | scaffold267:650,101-650,598 | AGTTGCGTCAGCAATTG | 242bp~250bp | no recombinant | h x h^c | h > |
| srp_3’-F           | scaffold267:180,579-180,597 | AGTTGCGTCAGCAATTG | 242bp~250bp | no recombinant | h x h^c | h > |
| srp_3’-R           | scaffold267:180,579-180,597 | AGTTGCGTCAGCAATTG | 242bp~250bp | no recombinant | h x h^c | h > |
| kis_3’-F           | scaffold267:465,373-465,708 | AGTTGCGTCAGCAATTG | 242bp~250bp | no recombinant | h x h^c | h > |
| kis_3’-R           | scaffold267:465,373-465,708 | AGTTGCGTCAGCAATTG | 242bp~250bp | no recombinant | h x h^c | h > |
| RAD-tag_5’_breakpoint | scaffold294:486,936 | C/- (466,936) | ~60bp/71bp | hA/hSp-#61 (~60bp/71bp heterozygote) | h^4 x h^sp | h^4 < h^sp |
|                     | scaffold294:486,936 | C/- (466,936) | ~60bp/71bp | hA/hSp-#61 (~60bp/71bp heterozygote) | h^4 x h^sp | h^4 < h^sp |
### Supplementary Table 3 | Statistics of the genome assemblies

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

| Species          | Sample | Assembler     | Input data                  | Total (bp)     | No. of sequences | N50 length (bp) | Gap rate (%) | Max length (bp) | Complete (%) | Duplicated & complete (%) | Fragmented (%) | Missing (%) |
|------------------|--------|---------------|-----------------------------|----------------|------------------|----------------|--------------|----------------|---------------|---------------------------|----------------|-------------|
| *H. axyridis*    | h² 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       |
| *H. axyridis*    | h² 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        |
| *H. axyridis*    | h² F2  | Supernova     | 10x linked-reads            | 510,576,820    | 50,277           | 57,217          | 4.5          | 6,058,326     | 95.838        | 7.539                     | 2.593          | 1.568       |
| *H. axyridis*    | h 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        |
| *C. septempunctata* | 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       |
### Supplementary Table 6 | Primers for gene cloning

| Primer name                  | Target direction | Sequence (5'>3')                                                                 |
|------------------------------|------------------|-------------------------------------------------------------------------------|
| pannier-F                    | sense            | ATIGAYTTYCARTTYGGIGA                                                          |
| pannier-R                    | antisense        | GGYTTICKYTTICKGTYTG                                                           |
| rp49-F                       | sense            | ACIAARMUYYTATMIGCA                                                           |
| rp49-R                       | antisense        | TGIGCIAYTCISRCARTA                                                            |
| Ha-pannier-RACE-1            | sense            | GGGCAGGGAGTGCGTCAATTGTGGGCG                                                  |
| Ha-pannier-RACE-2            | sense            | CCAACCCCTCTGTGGAGAGAGATGGTAC                                                  |
| Ha-pannier-RACE-3            | antisense        | GGCACAGGGCTGGACACAGGTTGCG                                                    |
| Ha-pannier-RACE-4            | antisense        | GATGCGTGCTCTTGCGCATGGCCAGGGG                                                 |
| Cs-pannier-RACE-1            | sense            | AATTGCGGCACCCGACCAAGACGACGCTC                                                 |
| Cs-pannier-RACE-2            | sense            | AAGCTGACGGCGGTCAACAGGCGGCTCG                                                  |
| Cs-pannier-RACE-3            | antisense        | ATACATGCTTTGCGCGGTAT                                                      |
| Cs-pannier-5                 | sense            | GATCCGAACTTAGCGGCGTG                                                        |
| Cs-pannier-6                 | antisense        | GGCTGCTTTCATCCATCATCTTGGT                                                    |
| Ha_pnr_intron_conserved1_F   | sense            | TCAGCRAATCTTCACAT                                                          |
| Ha_pnr_intron_conserved1_R   | antisense        | CTCACACTCTATTCAAT                                                          |
| Ha_pnr_intron_conserved2_F   | sense            | AGAGAAAAAGACAATTTG                                                          |
| Ha_pnr_intron_conserved2_R   | antisense        | AAAAGTCTTTCTTCAGG                                                         |
| Ha_pnr_intron_conserved3_F   | sense            | AATGKATCCAAACCCYGAC                                                        |
| Ha_pnr_intron_conserved3_R   | antisense        | MGRAAGCTGAAATGAAGG                                                          |

(I=inosine, K=G+T, M=A+C, R=A+G, S=C+G, Y=T+C)
| Primer name | Target direction | Sequence (5’>3’) |
|-------------|-----------------|-----------------|
| Ha-pannier-1 | sense           | TCGAGCCTGTTGAAAGCAGCGAACCAGG |
| Ha-pannier-2 | antisense       | GGGTCCTCGGACGAGATTGATCT |
| Ha-pannier-3 | sense           | GCTCCACCTCGTAGAAGAC |
| Ha-pannier-4 | antisense       | AGCCATCAGTTGGGAGAAG |
| Cs-pannier-1 | sense           | GCGCGTGAAGAGATGACAG |
| Cs-pannier-2 | antisense       | GCCATCAGTTAGGGACGC |
| Cs-pannier-3 | sense           | TGTTGACGGGCTAGTTACT |
| Cs-pannier-4 | antisense       | CTGTTGACGGGCCTGGACTT |
| Ha-rp49-1   | sense           | GCGATCGCTATGGAACCTC |
| Ha-rp49-2   | antisense       | TACGATTTGCATCAAAGGT |
| Cs-rp49-1   | sense           | AGTGATCGTTATGGGAAAGCT |
| Cs-rp49-2   | antisense       | TCTGATTTGCATCAAAGGAC |
**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        | TCCACCAAGAAATAAGGAAGAAATTGAGG | PCR-1         |
| Ha-pannier-seq1       | sense            | GGGCAGGGAGTGGCTCAATTGTGGGGCC | Sequencing-1   |
| Ha-pannier-seq2       | antisense        | GGTTCTGTTCACTCCATCATCTTGTTG | Sequencing-1   |
| Ha-pannier-seq3       | sense            | AAAACAGTGCTGTGTAGT          | Sequencing-1   |
| Ha-pannier-seq4       | antisense        | ATAAGGTGAGTCGTTGGAATCCAGA  | Sequencing-1   |
| Ha-pannier-seq5       | sense            | CGACAAGATGAATGGGATGAACAGACCC | Sequencing-1   |
| Ha-pannier-seq6       | antisense        | TCTTGCTCTGTATGCGT           | Sequencing-1   |
| Ha-pannier-5’-F       | sense            | AGTTCTTCAGCCCTCTAAAGITTCACAGA | PCR-2         |
| Ha-pannier-5’-R       | antisense        | GGTTCTGTTCACTCCTACCTTTGTG   | PCR-2         |
| Ha-pannier-seq7       | sense            | CATCGCTCTCCAGATTAGGTGTAACCGA | Sequencing-2   |
| Ha-pannier-seq8       | antisense        | ATAAGGTGAGTCCGGTGGGAATCCAGA | Sequencing-2   |
| Ha-pannier-exon3B-F1  | sense            | GTTTCCACCAACACCTTC          | PCR-3         |
| Ha-pannier-exon3B-R1  | antisense        | GTCTGGTGGTGAAGCTTTATT       | PCR-3         |
| Ha-pannier-seq9       | antisense        | GATGCAGCTCTGCTCTCGCAAGGG    | Sequencing-3   |
| Ha-pannier-exon3B-F2  | sense            | ACCTTATGAAATGATGACCA        | PCR-4         |
| Ha-pannier-exon3B-R2  | antisense        | GATAGCCTTTACTGTGTTCT         | PCR-4         |
| Ha-pannier-seq10      | sense            | TCTCCACCTCCTCGCGGGTACACAGTC | Sequencing-4   |
**Supplementary Table 9 | Primers for riboprobe synthesis (in vitro transcription, IVT)**

| Primer name          | Target direction | Sequence (5'-3')                                      | Use of primers |
|----------------------|------------------|-------------------------------------------------------|----------------|
| Ha-pannier-O R-F     | sense            | GTCAGCATGTTTCACACC                                    | ORF cloning    |
| Ha-pannier-O R-R     | antisense        | TCTTTGCTTTTTATGGT                                      | ORF cloning    |
| T3                   | sense            | ATTAACCTCCTAAAGGA                                     | IVT template (5' ORF) |
| SP6-Ha-pan-R-R       | antisense        | atttaggtgaacctagagagaAACAGGTGTGTGTAGT                 | IVT template (5' ORF) |
| T7                   | antisense        | TAATACGACTCATAGGG                                     | IVT template (3' ORF) |
| SP6-Ha-pan-CF        | sense            | atttaggtgaacctagagagaAACAGGTGTGTGTAGT                 | IVT template (3' ORF) |
Supplementary Figure 1 | Snapshots of pharate adult elytral pigmentation in *H. axyridis*

Melanin synthesis activity (black) and carotenoids accumulation (orange) were simultaneously visualised using *hC* elytra stained with PO activity. Strong black and orange signals appeared after 80 h AP.

Supplementary Figure 2 | Expression analysis of *H. axyridis (Ha) pannier* in elytral primordia by RT-PCR.

a, Developmental expression profiles of *pannier* from the final instar larvae to pupae. *Ha-pnr, Ha-pannier, Ha-rp49, Ha-ribosomal protein 49* (internal control). Adult emergence is at 4.5 days AP in our rearing condition. Days after the onset of the each stage are indicated above. (b, b’) Spatial distribution of *pannier* at 84 h AP. Future black (B1-B3) and red (R) regions were isolated for RT-PCR. Pharate adult elytra of three individuals were analysed (#1-#3). *rp49*, internal control. b’, Left panel, pharate adult elytra with 3 hour PO staining (84 h AP). Right panel, an example of an elytron after isolation of red and black regions. Scale bars, 1 mm.
Supplementary Figure 3 | Validation of the pannier-locus scaffold of Platanus2 for the *H. axyridis* F2-3 sample

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

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

**a–d,** The dot plots between the consensus scaffolds obtained by reassembly of F2-3 mate-pair reads with Platanus2, and each consensus scaffold obtained by *de novo* genome assembly of the 10x linked-reads. **a,** \( h^C (F2-3) \) vs. \( h^C (NT6) \). **b,** \( h^C (F2-3) \) vs. \( h^A \) (F2-hybrid). **c,** \( h^C (F2-3) \) vs. \( h \) (NT8). **d,** \( h^C (F2-3) \) vs. *C. sep.* The colour code for colouring the homologous segments is on the right side of each panel. Blue arrow, the *pannier* locus. The green and the magenta pins indicate the position of the breakpoint.
genotyping markers located on the outermost side, of all genotyping markers found to show association with the $h$ locus in the three crossing experiments ($mbl$ and $Mink$ in Supplementary Table 2, respectively). The subset regions of each scaffold between these two markers or the corresponding regions were extracted for dot plot analyses. In each pair of comparison, sequential linear homology between the two scaffolds was confirmed.

The responsible region of each $h$ allele ($h^C$, 690 kb; $h^A$, 660 kb; $h$, 2.1 Mb + $\alpha$) was included in a single linked-read genomic scaffold (a–c). In *C. septempunctata*, the genomic region corresponding to the region just downstream of the *pannier* locus in *H. axyridis*, was located in the scaffold different from that including *pannier* (scaffold 47 and 92), implying at least one translocation event in either of the two ancestral lineages (d).

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

a, Nucleotide polymorphisms in *pannier* ORF in 4 allelic strains of $h$ ($h^A$, $h^{Sp}$, $h^C$). $R = G$ or $A$, $Y = T$ or $C$, $M = A$ or $C$, $S = G$ or $C$, $W = A$ or $T$. b, Amino acid sequences deduced
from ORF sequences in (a). X = T or S. The alternative exon region (exon 3A) and GATA zinc finger domains are indicated with grey and yellow, respectively.

Supplementary Figure 6 | Polymorphism in ORF sequences of pannier-B isoform in the 4 allelic strains of the h locus.

a, Nucleotide polymorphisms in pannier ORF in 4 allelic strains of h (h, h^A, h^Sp, h^C). R = G or A, Y = T or C, M = A or C, S = G or C, W = A or T. b, Amino acid sequences deduced from ORF sequences in (a). X = T or S. The alternative exon region (exon 3B) and GATA zinc finger domains are indicated with grey and yellow, respectively.

Supplementary Figure 7 | vestigial mRNA is upregulated in the presumptive black regions from early pupal stages in the h^C background.

1B, black region at 24 h AP. 1R, red region at 24 h AP. 3B, black region at 72 h AP. 3R, red region at 72 h AP. Read count of each sample was extracted from RNA-seq analysis data. Bars, mean read counts. (n = 3). Error bars, standard error of means. *, FDR < 0.01.
Supplementary Figure 8 | Expression analysis of C. septempunctata (Cs) panner in elytral primordia by RT-PCR.

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

Cs-rp49, Cs-ribosomal protein 49 (internal control). Adult emergence is at 4.5 days AP in our rearing condition. Days after the onset of the each stage are indicated above. b, b’,

Spatial distribution of panner at 84 h AP. Future black (B1–B2) and red (R1–R3) regions were isolated for RT-PCR. Pharate adult elytra of three individuals were analysed (#1–#3). Cs-rp49, internal control. b’, Left panel, pharate adult elytra with 3 hour PO staining (84 h AP). Right panel, an example of an elytron after PO staining for 1 hour and isolation of red and black regions. Scale bars, 1 mm.

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

a, The aligned nucleotide sequences of panner ORF before trimming gapped and shifted regions. b, The aligned nucleotide sequences of panner ORF after trimming. c, The aligned nucleotide sequences of the conserved intronic regions of panner (concatenated three blocks) before trimming gapped and shifted regions. d, The aligned nucleotide
sequences of the conserved intronic regions of pannier after trimming. A dash indicates a

gap.
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Author contributions

T.N. and T.A conceived this study. T.M. and T.N. analysed the elytral pigmentation processes. T.A., T.M., K.G., K.H., A.I. and J.Y. analysed the sequence data. T.A, T.M., K.G., A.I., K.H., and J.H. performed cloning of the pannier genes from different alleles and species of ladybirds. T.M., K.G., A.I., K.H., and J.H. performed the larval RNAi experiments. K.H., K.G. and J.H. performed the semi-quantitative RT-PCR. T.A. performed the in situ hybridisation. J.Y. collected the total RNA for the RNA-seq analysis, and the genomic DNA samples for the initial de novo genome assembly. T.A. collected the DNA samples for the resequencing analyses. M.S. and Y.S. collected the RNA-seq raw data. Y.M. and A.T. performed the initial de novo genome assembly. K.Y. and S.S. collected the raw data for the resequencing and the RAD-seq analyses. K.Y. constructed the flexible ddRAD-seq protocol. R.K, M.O, and T.I. performed reassembly of the genome, de novo assembly of the linked-read genomic data, and validation of the obtained genomic scaffolds. M.K., T.T., T.A. and K.Y. performed mapping and quantification of the RNA-seq data. T.A. performed the data analyses for the genetic association studies, the gene annotation, the motif enrichment analysis, and the molecular
phylogenetic analyses around the *pannier* locus. T.A and T.N. wrote, and all authors commented on the manuscript.
DNA Data Bank of Japan (DDBJ) accession numbers for the sequences reported in this article are LC269047–LC269055 for Ha-pnr, LC269056 for Cs-pnr and LC269057 for Cs-rp49, DRA002559 for the genomic sequencing data of H. axyridis, DRA006068 for the genomic resequencing data of H. axyridis, and DRA005777 for the RNA-seq data. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature.

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