DIVISION OF EVOLUTIONARY DEVELOPMENTAL BIOLOGY

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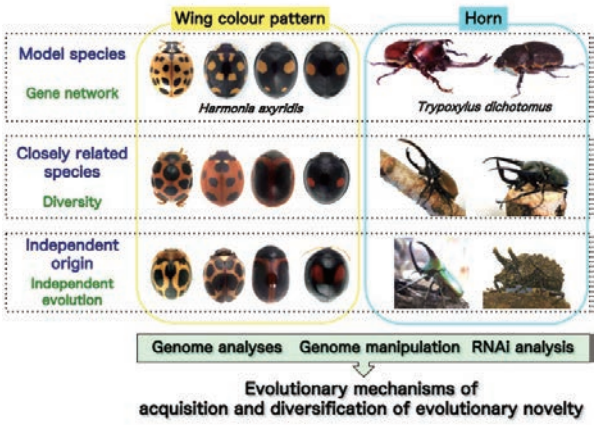
The Division of Evolutionary Developmental Biology was started in June 2015. We focus on the evolutionary novelties acquired by insects in order to elucidate the molecular and evolutionary mechanisms that lead to the large variety of traits that they display. Among this wealth of exciting traits, our lab is currently focused on promoting research into (1) the origin and diversification of insect wings, (2) wing color patterns and mimicry of ladybird beetles, and (3) the acquisition and diversification of beetle horns.



Visual overview of this lab's work.

I. Origin and diversification of insect wings

The flight organs of insects have uniquely evolved when compared to that of other various flying animals on earth. Despite more than two centuries of debate, the evolutionary origin of insect wings is still an enigma; one which we are trying to decipher by the use of evo-devo methods. In *Drosophila melanogaster*, the wing master gene *vestigial* (*vg*) and its interaction partner *scalloped* (*sd*) play pivotal roles in the formation of wing field identity. For this reason, these genes are ideal research candidates in the investigation of wing origin and evolution.

One way to identify the structure from which insect wings first evolved is to explore the function of "wing" genes in ancestral wingless (apterygote) species. To achieve this end, we chose the firebrat, *Thermobia domestica*, as a model (Figure 1A). *T. domestica* belongs to Thysanura, which is phylogenetically the closest extant relative of winged (pterygote) insects, thus making it ideal for elucidating wing origin. We cloned *vg* and *sd* orthologs from *T. domestica* (*Td-vg* and *Td-sd*), and developed RNA interference (RNAi) based methods for *T. domestica* to examine the functions of these genes. We are currently testing the functional effects of altered transcription for each of these wing genes in ancestrally wingless firebrats. Furthermore, we are performing comparative analyses of the function of these same genes in "primitively winged" (hemimetabolous) insects (Figure 1B) to obtain additional clues relevant to us understanding the origin and evolution of insect wings.

Figure 1. The firebrat, *Thermobia domestica* (A). The two-spotted cricket, *Gryllus bimaculatus* (B).

Interestingly, our previous work showed that *vg* expressing epidermal tissue forms lateral outgrowths in non-winged segments in the mealworm beetle (Ohde *et al.*, 2013). Based on this, we hypothesize that ancestral lateral body wall outgrowths evolved into functional wings. However, genetic tools available for the analysis of basally branching wingless species are limited. To overcome these limitations, we established CRISPR/Cas9-based germline genome editing in *T. domestica*. Heritable mutations were successfully introduced in *white* locus, an evolutionarily conserved gene, encoding the ATP-binding cassette (ABC) membrane transporter, of *T. domestica* by using CRISPR/Cas9 system. This in turn results in white-eyed firebrats. In addition to the RNAi-mediated gene knockdown (Ohde *et al.*, 2009), germline genome editing using CRISPR/Cas9 in *T. domestica* provides a platform technology for creating new research opportunities concerning the evolution of insects, such as insect wing origin. We are now conducting gene knock-out/in within various "wing" genes to identify genetic details and cell lineage analyses in *T. domestica* (Figure 1).

II. Wing color patterns and mimicry of ladybird beetles

A tremendous range of diversity in wing color patterns has evolved among insects, which in turn plays various ecologically important roles such as intraspecific sexual signaling, mimesis, mimicry, and is also used as a warning signal to predators. However, the molecular mechanisms responsible for generating such color patterns in most ladybird species remain elusive. To investigate the developmental mechanisms of color pattern formation, we have been focusing on the multicolored Asian ladybird beetle, *Harmonia axyridis*, which has conspicuous and variable wing color patterns consisting of black and red pigments (Figure 2A). The ladybird's vivid wing color pattern acts as a warning signal to predators that they taste bad. At the same time, various other insect species utilize this ecological signal by mimicking the ladybirds' wing color patterns. Mimicry provides us with an exciting opportunity to study how independent lineages of insects have evolved convergent color patterns. To explore color pattern formation mechanisms in mimicry, we are currently focusing on the leaf beetle, *Argopistes coccinelliformis*, which has color patterns similar to *Harmonia*, and is thought to be a Batesian mimicry of ladybird beetles (Figure 2B). To elucidate the molecular mechanisms underlying these wing color patterns, we established a technique for germline transformation using a *piggyBac* vector and RNAi in the ladybirds.

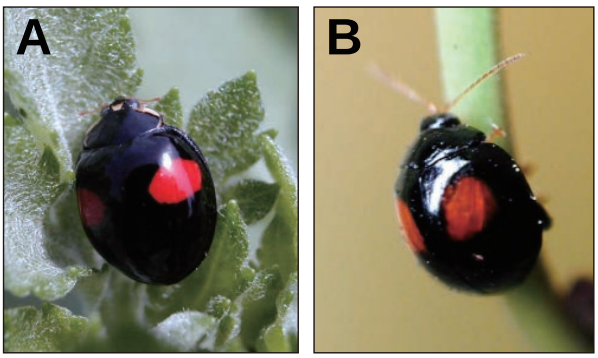


Figure 2. The ladybird beetle, *Harmonia axyridis* (A) and the leaf beetle, *Argopistes coccinelliformis* (B).

We recently identified a key gene, *pannier*, which regulates intraspecific color pattern polymorphism in *H. axyridis* using next generation sequencing technologies (RNA-seq and *de novo* genome assembly), and an RNAi-based screening method that we have established. *pannier* is expressed in specific regions in the wing, which synthesizes black pigment, and suppresses red pigmentation. The expression pattern of *pannier* is diversified according to the diverse color pattern types in *H. axyridis*. These findings suggest that regulatory shift, such as changes in enhancer activity, at the *pannier* locus may be crucial for the evolution of wing color patterns in *H. axyridis*. We are currently trying to elucidate the evolutionary origin of color patterns in ladybirds with a focus on regulatory shifts at the *pannier* loci.

We are also attempting to apply genome-editing technologies such as TALEN and CRISPR/Cas9 to tackle this issue. Thus far, we have achieved an efficient method of gene disruption using TALEN. Recently, we have begun successfully establishing the disrupting *pannier* gene function using CRISPR/Cas9 system. Ribonucleoprotein (RNP) complex of Cas9 protein and guide RNA targeting *pannier* coding exon was microinjected into the fertilized eggs. As a result, over 60% of G0 founders produced *pannier* knockout progenies (Figure 3). CRISPR/cas9 mediated gene disruption has advantages in terms of ease of designing gRNA and also possesses a high KO efficiency compared to TALEN mediated gene modification in *Harmonia*. To this end, we are continuing to develop CRISPR/Cas9 mediated gene knock-in method. To apply this approach, we are trying to attempt more complicated genome editing techniques such as genomic insertion, inversion and duplication to identify the crucial regulatory shift that may have driven the evolution of wing color patterns in ladybird beetles.

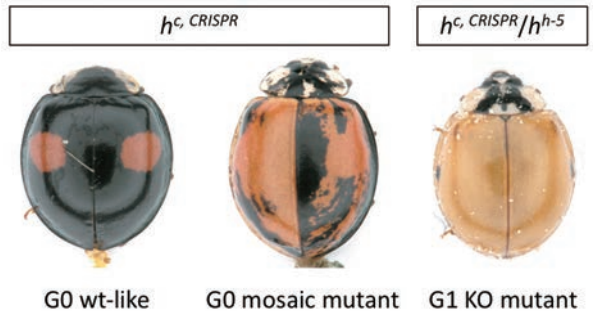


Figure 3. Representative photographs of *pannier* mutant phenotypes in G0 wt-like (left side), G0 mosaic (middle side) and G1 KO mutant (right side).

We are also establishing cryopreservation methods for germline cells in ladybird beetles to assist us with this. This is due to the high risk of losing valuable genetic bioresources in non-model insects. We recently established ovary transplantation and ovarian cryopreservation techniques in ladybird beetles. We hope that the genetic tools and techniques that we have established will further facilitate this research.

We plan to eventually analyze how the similar wing color patterns of model and mimic are generated based on the knowledge obtained from *H. axyridis*. For example, do they use conserved or divergent mechanisms?

III. Acquisition and diversification of beetle horns

Insects show a tremendous range of diversity in "horns"; rigid body outgrowths that function as weapons. Horns are a subject of great potential for evo-devo studies because they have arisen multiple times *de novo*, as evolutionary "novelties". However, the molecular mechanisms involved in sexually dimorphic horn formation are still poorly understood. To investigate the developmental mechanisms of horn formation, we are focusing on the Japanese rhinoceros beetle, *Trypoxylus dichotomus* (Coleoptera), which exhibits remarkable sexual dimorphisms in head and thoracic horns. The male-specific horns of *T. dichotomus* are among the best models for studying how an extreme, sex-specific morphology is formed (Figure 4, Control).

We have recently developed a larval RNAi technique for

T. dichotomus, which has allowed us to molecularly dissect the relationship between the conserved genetic pathway for sex differentiation and sexually dimorphic horn formation during post embryonic development. We systematically evaluated the function of the sex determination gene, *transformer* (*tra*) in different developmental stages, and revealed in which tissue and developmental stage the gene regulatory network for sex differentiation is activated to form sexual dimorphic horns in the head and thorax. In *T. dichotomus*, *tra* regulates sex-specific splicing of the *doublesex* pre-mRNA, and its loss of function results in sex transformation in females (Figure 4). *tra* RNAi treatments in females at early developmental stages during metamorphosis resulted in full sexual transformation, whereas no transformation is observed in the treatments at later stages. Therefore, we were able to estimate the onset of activation of the developmental program for the sexually dimorphic horn formation by determining the latest RNAi treatment timing when a full sexual transformation phenotype is observed. Based on this approach, we estimated that the developmental program for sexually dimorphic horn formation is activated at 29 hours after the prepupal period.

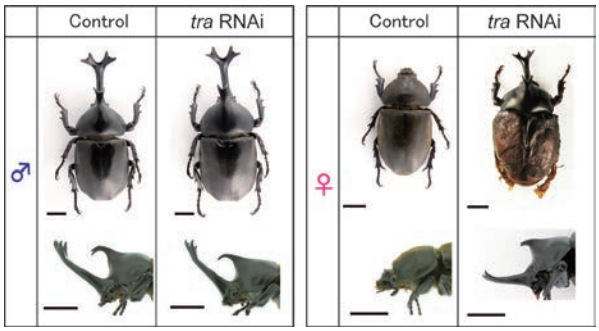


Figure 4. *tra* RNAi phenotypes. In the *tra* RNAi females, ectopic horn formation was caused by sex transformation (masculinization).
(Adapted from Morita *et al*., PLOS Genet., 15: e1008063, 2019)

We are currently focusing on this developmental stage because crucial regulatory factors for horn formation and differentiation are supposed to be activated at this stage in *T. dichotomus*. The present study provides a good starting point in unveiling the gene regulatory network for sexually dimorphic horn formation and to pursue the evolutionary origin of such a regulatory system.

Publication List:

〔Original papers〕

- Adachi, H., Matsuda, K., Niimi, T., Kondo, S., and Gotoh, H. (2020). Genetical control of 2D pattern and depth of the primordial furrow that prefigures 3D shape of the rhinoceros beetle horn. Sci. Rep. *10*. DOI: 10.1038/s41598-020-75709-y
- Chikami, Y., Kawaguchi, H., Suzuki, T., Yoshioka, H., Sato, Y., Yaginuma, T., and Niimi, T. (2021). Oral RNAi of *diap1* results in rapid reduction of damage to potatoes in *Henosepilachna vigintioctopunctata*. J. Pest Sci. *94*, 505–515. DOI: 10.1007/s10340-020-01276-w
- Sakai, H., Konagaya, T., Takemura, Y., Sahara, K., and Niimi, T. (2020). Double-copulated introduction of ejaculate with dominant larval phenotype to maintain *Bombyx mori* mutant with dysfunctional apyrene sperm. J. Insect Biotechnol. Sericology *89*, 2_039-2_043. DOI: 10.11416/jibs.89.2_039