

## DIVISION OF EVOLUTIONARY DEVELOPMENTAL BIOLOGY



Professor  
NIIMI, Teruyuki

Assistant Professor: ANDO, Toshiya  
NAKAMURA, Taro

Specially Appointed Assistant Professor:  
MORITA, Shinichi

Technical Staff: MIZUTANI, Takeshi

Postdoctoral Fellow: MATSUOKA, Yuji  
SAKURA, Kazuki

JSPS Postdoctoral Fellow: YANO, Koki

Research Staff: CHIKAMI, Yasuhiko  
KAWAGUCHI, Haruka  
MORITA, Junko  
TAGUCHI, Rie

SOKENDAI Graduate Student: KITAZAWA, Yurina

Visiting Undergraduate: ODA, Hiroki

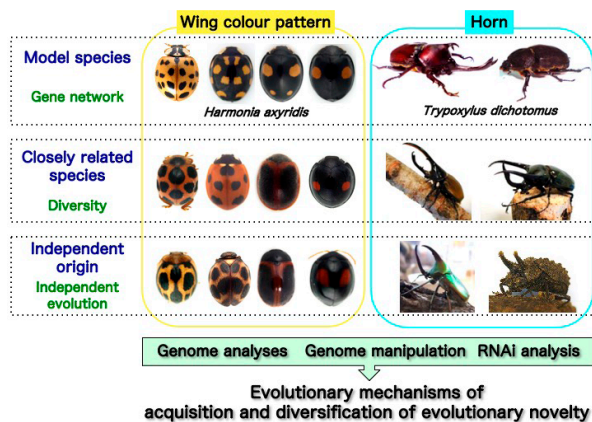
Visiting Scientist: TAKENAKA, Masaki

Technical Assistant: YOKOYAMA, Michiko  
SUGIYAMA, Hiroko

Admin Support Staff: SAITO, Eiko



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.



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

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.

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.

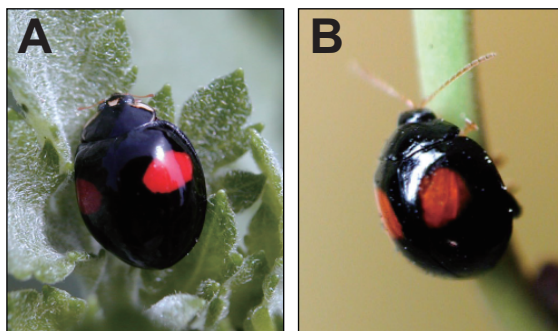


Figure 2. The ladybird beetle, *Harmonia axyridis* (A) and the leaf beetle, *Argopistes coccinelliformis* (B).

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.

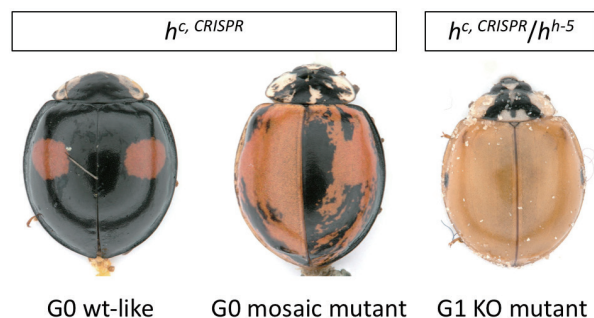


Figure 3. Representative photographs of *pannier* mutant phenotypes in G0 wt-like (left side), G0 mosaic (middle side) and G1 KO mutant (right side).

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 *de novo* multiple times 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 morphological structure is formed (Figure 4 A).

We first investigated the process of horn primordium formation to understand the developmental mechanism of horn formation. The insect body is covered by a rigid exoskeleton mainly consisting of chitin, so changes in body size and shapes are produced by molting. Beetle horns first appear at the pupal period (Figure 4 A). During the prepupal period (about 130 hours) which is an intermediate stage between the last larval instar stage and pupation, the head horn primordia are packed folded inside the larval head capsule (Figure 4 B). This folded horn primordium develops into its final 3D shape (pupal horn) during pupation. To properly make the horn during pupation, the folding pattern (Figure 4 B) and surface micro furrows (Figure 4 C) are precisely formed on the horn primordium and packed within the confined space of the prepupal head. We recently attempt to use micro-CT to analyze 3D pattern formation of head horn primordia during the prepupal period. We found that horn 3D morphogenesis is accomplished in only 72 hours from the prepupal initiation (Figure 4 B, C).

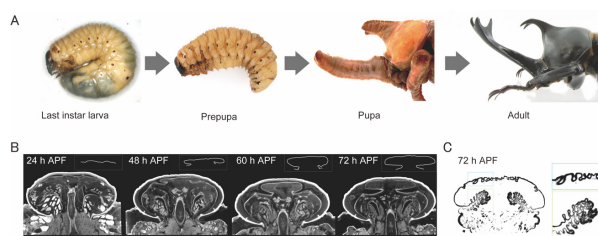


Figure 4 Morphological change of horn primordia in *Trypoxylus dichotomus*.

(A) Developmental process of *Trypoxylus dichotomus* male. (B, C) Micro-CT images of the male head horn primordium during pupal development. (Adapted from Morita and Niimi, *Sanshi Konchu Biotec*, 86: 145–151, 2020.)

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 to unveil the gene regulatory network for sexually dimorphic horn formation and to pursue the evolutionary origin of such a regulatory system.

## Publication List:

### [Original papers]

- Hashiro, S., Chikami, Y., Kawaguchi, H., Krylov, A.A., Niimi, T., and Yasueda, H. (2021). Efficient production of long double-stranded RNAs applicable to agricultural pest control by *Corynebacterium glutamicum* equipped with coliphage T7-expression system. *Appl. Microbiol. Biotechnol.* 105, 4987–5000. DOI: 10.1007/s00253-021-11324-9
- Miyazaki, S., Fujiwara, K., Kai, K., Masuoka, Y., Gotoh, H., Niimi, T., Hayashi, Y., Shigenobu, S., and Maekawa, K. (2021). Evolutionary transition of *doublesex* regulation from sex-specific splicing to male-specific transcription in termites. *Sci. Rep.* 11, 15992. DOI: 10.1038/s41598-021-95423-7
- Ohde, T., Mito, T., and Niimi, T. (2022). A hemimetabolous wing development suggests the wing origin from lateral tergum of a wingless ancestor. *Nat. Commun.* 13, 979. DOI: 10.1038/s41467-022-28624-x
- Ylla, G., Nakamura, T., Itoh, T., Kajitani, R., Toyoda, A., Tomonari, S., Bando, T., Ishimaru, Y., Watanabe, T., Fuketa, M., Matsuoaka, Y., Barnett, A.A., Noji, S., Mito, T., and Extavour, C.G. (2021). Insights into the genomic evolution of insects from cricket genomes. *Commun. Biol.* 4, 733. DOI: 10.1038/s42003-021-02197-9

### [Review article]

- Niimi, T., and Ando, T. (2021). Evo-devo of wing colour patterns in beetles. *Curr. Opin. Genet. & Dev.* 69, 97–102. DOI: 10.1016/j.gde.2021.02.007