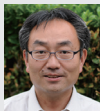


DIVISION OF EVOLUTIONARY DEVELOPMENTAL BIOLOGY



Professor
NIIMI, Teruyuki

Assistant Professor:	ANDO, Toshiya NAKAMURA, Taro
Technical Staff:	MIZUTANI, Takeshi
NIBB Research Fellow:	MORITA, Shinichi
JSPS Postdoctoral Fellow:	SAKAI, Hiroki KONAGAYA, Tatsuro
Postdoctoral Fellow:	KAWAGUCHI, Haruka
SOKENDAI Graduate Student:	CHIKAMI, Yasuhiko FUJIKAKE, Yuma
Visiting Undergraduate:	HORI, Kiyoshiro
Technical Assistant:	MORITA, Junko HACHISUKA, Yukari
Secretary:	SAITO, Eiko KIYOHARA, Megumi

The Division of Evolutionary Developmental Biology was started in June 2015. We focus on the evolutionary novelties acquired by insects through evolution in order to elucidate the molecular and evolutionary mechanisms that lead to the large variety of traits that they display. From 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) acquisition and diversification of beetle horns.

I. Origin and diversification of insect wings

Of the various flying animals on the earth, the flight organ of insects has uniquely evolved. Despite over two centuries of debate, the evolutionary origin of the insect wing is still an enigma. We are trying to uncover it by using 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 candidates for investigating 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. We chose the firebrat, *Thermobia domestica*, as a model (Figure 1A). *T.*



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

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 we developed RNA interference (RNAi) based methods for *T. domestica* to examine the functions of these genes. We are currently testing for the functional effects of altered transcription of each of these wing genes in the ancestrally wingless firebrats. In addition, 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 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 these facts, 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, which 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 that opens new research opportunities on the evolution of insects, such as on the insect wing origin. We are now conducting gene knock-out/in within various “wing” genes to identify details genetic and cell lineage analyses in *T. domestica* (Figure 1).

II. Wing color patterns and mimicry of ladybird beetles

A tremendous range of diversity of wing color patterns have evolved within insects, which play various ecologically important roles such as intraspecific sexual signaling, mimesis, mimicry, and warning against 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

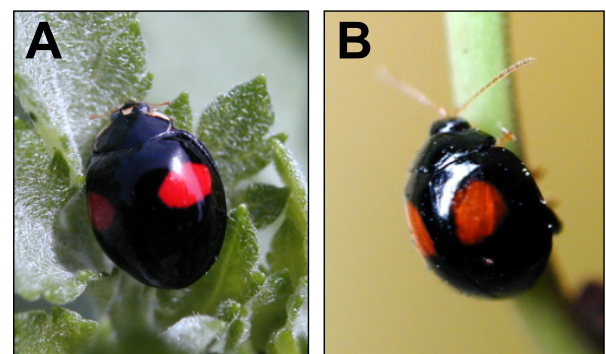


Figure 2. The ladybird beetle, *Harmonia axyridis* (A) and the leaf beetle, *Argopistes coccinelliformis* (B).

and variable wing color patterns consisting of black and red pigments (Figure 2A). The ladybird's vivid wing color pattern functions 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 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 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.

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 established. *pannier* is expressed in specific regions in the wing, which will synthesize black pigment, and suppress 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 change 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 focusing on regulatory shifts at the *pannier* loci. We are also establishing genome-editing technologies using TALEN and CRISPR/Cas9 to tackle this issue, and have already established an efficient method of gene disruption. At present, we are establishing more complicated genome editing techniques such as genomic insertion, inversion and duplication to identify the crucial regulatory shift that may have driven evolution of wing color patterns in ladybird beetles. We are also establishing cryopreservation methods for germline cells in ladybird beetles to overcome laborious tasks and 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 facilitate the ladybird beetle's research.

In the future, we are planning to 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 one of the best models to study how an extreme, sex-specific morphology is formed (Figure 3, Control). We have developed a technique for larval RNAi in *T. dichotomus*, allowing us to rigorously and systematically test the functional roles of a large suite of candidate developmental genes, revealing for the first time the molecular mechanisms responsible for the growth of male rhino beetle horns. In addition, we are employing a high throughput approach. To identify novel genes involved in the sexually dimorphic horn development in *T. dichotomus*, the developing horn discs' mRNA has been assessed by deep-sequencing transcriptome analysis (RNA-seq). We narrowed down the genes associated with horn formation to 49 genes, and performed RNAi-based knockdown screening to provide deep insights into where, when, and how the head and thoracic horns are formed during development. We successfully identified 11 transcription factors (*SP8*, *pannier*, *Rx*, *Optix*, *BarH1*, *Tbx20*, *Sox21b*, *dachshund*, *Scr*, *Sox14*, *abrupt*) that contribute to horn formation. These 11 genes include larval head- and appendage-patterning genes, which are involved in *Onthophagus* horn formation, suggesting the early redeployment of this subset of genes during the scarab horn evolution (Figure 3).

To understand how beetle horns have molecularly diversified, we are extending our analyses to include additional beetle species with different types of exaggerated horns, including rhinoceros beetles with diverse horn structures as well as horned beetles in other phylogenetic groups.

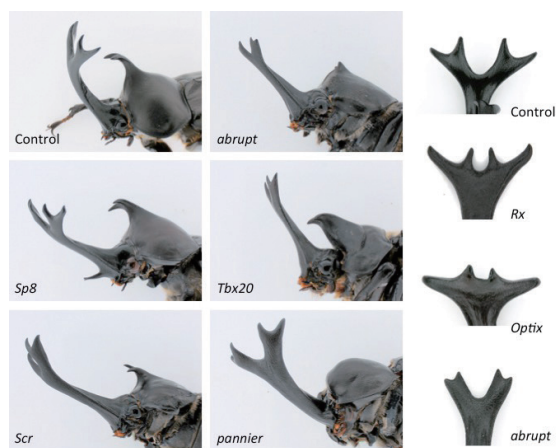


Figure 3. RNAi-mediated gene knockdown alters horn shapes and sizes in head and thorax of the Japanese rhinoceros beetle, *Trypoxylus dichotomus*.

Publication List:

[Original papers]

- Adachi, H., Matsuda, K., Niimi, T., Inoue, Y., Kondo, S., and Gotoh, H. (2018). Anisotropy of cell division and epithelial sheet bending via apical constriction shape the complex folding pattern of beetle horn primordia. *Mech. Dev.* 152, 32-37.
- Ando, T., Matsuda, T., Goto, K., Hara, K., Ito, A., Hirata, J., Yatomi, J., Kajitani, R., Okuno, M., Yamaguchi, K., Kobayashi, M., Takano, T., Minakuchi, Y., Seki, M., Suzuki, Y., Yano, K., Itoh, T., Shigenobu, S., Toyoda, A., and Niimi, T. (2018). Repeated inversions within a *pannier* intron drive diversification of intraspecific colour patterns of ladybird

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- Hust, J., Lavine, M.D., Worthington, A.M., Zinna, R.A., Gotoh, H., Niimi, T., and Lavine, L.C. (2018). The Fat-Dachsous signaling pathway regulates growth of horns in *Trypoxylus dichotomus*, but does not affect horn allometry. *J. Insect Physiol.* *105*, 85-94.
 - Kawaguchi, H., and Niimi, T. (2018). A method for cryopreservation of ovaries of the ladybird beetle, *Harmonia axyridis*. *J. Insect Biotechnol. Sericol.* *87*, 35-44.
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 - Ohde, T., Morita, S., Shigenobu, S., Morita, J., Mizutani, T., Gotoh, H., Zinna, R.A., Nakata, M., Ito, Y., Wada, K., Kitano, Y., Yuzaki, K., Toga, K., Mase, M., Kadota, K., Rushe, J., Lavine, L.C., Emlen, D.J., and Niimi, T. (2018). Rhinoceros beetle horn development reveals deep parallels with dung beetles. *PLoS Genet.* *14*, e1007651.
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 - Tsuji, T., Gotoh, H., Morita, S., Hirata, J., Minakuchi, Y., Yaginuma, T., Toyoda, A., and Niimi, T. (2018). Molecular characterization of eye pigmentation-related ABC transporter genes in the ladybird beetle *Harmonia axyridis* reveals striking gene duplication of the *white* gene. *Zool. Sci.* *35*, 260-267.
 - Zinna, R.A., Emlen, D.J., Lavine, L.C., Johns, A., Gotoh, H., Niimi, T., and Dworkin, I. (2018). Sexual dimorphism and heightened conditional expression in a sexually selected weapon in the Asian rhinoceros beetle. *Mol. Ecol.* *27*, 5049-5072.

[Review article]

- Zinna, R., Gotoh, H., Kojima, T., and Niimi, T. (2018). Recent advances in understanding the mechanisms of sexually dimorphic plasticity: Insights from beetle weapons and future directions. *Curr. Opin. Insect Sci.* *25*, 35-41.