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Author(s)	童, 欣
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博士論文要約

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Evolutionary ecology in gall-forming aphids: extreme polyphenism and biased sex ratios
（虫こぶ形成アブラムシの進化生態学：極端な表現型可塑性と偏った性比）

Aphids of the subfamily Eriosomatinae are characterized by complex life cycle, gall formation, host alternation, and seasonal polyphenism. The eriosomatine aphids induce galls on the leaves of *Ulmus* trees in spring, and migrate to the roots of secondary host plants, such as grasses and shrubs, where they reproduce asexually underground. Winged aphids emerge in autumn and return to *Ulmus* trees to reproduce sexual-generation offspring. The aphids in gall generation, the secondary-host generation, and the sexual generation are morphologically and physiologically distinct, even among aphids in that a variety of phenotypes (morphs) emerge within a clonal lineage. Galls induced by eriosomatine aphids are species-specific in order to meet different biological characters and this mechanism remains largely unknown. Moreover, as in other aphids, the aphids and their obligate intracellular endosymbiont *Buchnera aphidicola* maintain an extremely close symbiosis, for example, *Buchnera* can provide aphids with essential amino acids that are usually limited in phloem sap. In my research, I study eriosomatine aphids with conspicuous polyphenism and galling behavior, to elucidate the specificity of this group from various perspectives in genetics, physiology, and evolutionary ecology.

In Chapter 1, I investigated the roles of aphid endosymbionts in the three-way symbioses among plants, aphids, and aphid endosymbionts in galling system by applying high-quality genome sequencing on *Buchnera* from three gall-forming aphids *Tetraneura nigriabdominalis*, *Tetraneura sorini*, and *Eriosoma harunire*, which share same host plant *Ulmus davidiana* var. *japonica* to form galls on.

Genome sequencing of *Buchnera* of three gall-forming aphids was conducted using methods of Nanopore long-read sequencing and corrected by shotgun data generated by Illumina TruSeq. The library preparation was followed manufactures' instruction respectively. Libraries for Nanopore long reads were sequenced on GridION by Oxford Nanopore Technologies to generate long reads of genomes, and pooled libraries for Illumina TruSeq were sequenced on Illumina NextSeq 550 System to generate shotgun metagenomic sequence data of around 350 bp fragmented by Covaris Focused-ultrasonicator M220 at Functional Genomics Facility, National Institute for Basic Biology, Okazaki, Japan.

Nanopore long reads were mapped, assembled, and corrected by using Minimap2 (Li 2018), Miniasm (Li 2016), and Racon (Vaser *et al.* 2017), respectively. After

correction by Racon, Nanopore linked long reads were polished using Pilon version 1.24 (Walker *et al.* 2014) with adapter-trimmed paired-reads generated by Illumina TruSeq after quality check by FastQC v0.11.9 (Andrews 2010). Assembled contigs were used to generate a BLAST database against reference bacterial genomes of *Buchnera* in *Acyrthosiphon pisum* (Shigenobu *et al.* 2000) and *Baizongia pistaciae* (van Ham *et al.* 2003) to detect *Buchnera* genomes and their plasmids. The complete circle genomes were confirmed by manually recircling the contigs and mapped by both raw Nanopore linked long reads using Minimap2 and polished again using Pilon v.1.24 by Illumina paired reads for the final scaffolds. The genomes of *Buchnera* in *T. sorini*, *T. nigriabdominlis*, and *E. harunire* aphids were annotated using Prokka v.1.14.6 (Seemann 2014).

As a result, *Buchnera* genome sizes of *T. sorini*, *T. nigriabdominalis* and *E. harunire* were obtained as 533,871 bp, 530,863 bp and 627,315 bp, respectively, and the numbers of the protein-coding regions were 485, 474 and 489, respectively. *Buchnera* is found to be one single circle chromosome without any plasmids in three eriosomatine aphids. Genome annotation analysis also revealed that *leuABCD* genes that normally locate on plasmids of *Buchnera* (e.g. in *Acyrthosiphon pisum*) are found on the chromosomes. In addition, the *Buchnera* of *E. harunire* lacks the genes for biosynthesis of ornithine, which is an important precursor for the biosynthesis of the essential amino acid arginine. *E. harunire* may consume ornithine/arginine from the galls, or from other pathways (e.g. to synthesize ornithine by aphids) in which there is close tripartite symbiosis in galling system on nutritional compensation.

In Chapter 2, I explored the relationship between seasonal polyphenism and cuticular hydrocarbon profiles in the eriosomatine aphid, *Prociphilus oriens*. This species undergoes host alternation and exhibits five major morphs. These morphs are related to the differences between asexual and sexual generations, the difference in host plants, and the presence or absence of symbiotic ants.

The CHC extracts were analyzed using gas chromatography–mass spectrometry (GC-MS). The GC-MS system (Varian/CP-3800 and Varian/1200L, Varian Medical Systems, Inc., Palo Alto, California, USA) was equipped with the TC-5 column (30 m × 0.25 mm ID, 0.25 μm film; GL Sciences, Shinjuku, Tokyo, Japan). Temperature was kept at 100°C for 2 min, then increased by 40°C/min to 200°C, by 20°C/min to 260°C, by 10°C/min to 305°C, and finally by 5°C/min to 325°C. Helium was used as carrier gas with a constant flow of 1.8 mL/min. Analyses were run in a splitless mode with an injector temperature of 300°C. Electron ionization mass spectra were recorded with an ionization voltage of 70 eV and an ion source temperature of 250°C. Components were identified by their characteristic mass spectral fragmentation patterns and retention times.

Peaks detected by GC-MS were identified and the amounts of hydrocarbons were analyzed. Several kinds of alcohol were detected but removed from the analysis. Of the 28 hydrocarbons detected, I removed three hydrocarbons that are possessed by single

morphs only at a proportion of less than 1%, and subsequently the relative amounts of the remaining 25 hydrocarbons were normalized such that their total summed up to 100 (Table 2). I undertook principle component analysis based on the correlation matrix using the normalized relative proportions of hydrocarbons. All statistical analyses were carried out using JMP version 13 (SAS Institute, Cary, NC, USA).

In *P. oriens*, linear alkanes constituted the major proportion of cuticular hydrocarbons, but there were differences in their composition among morphs. In the morphs attended by ants, the ratio of *n*-C25 was higher than that of *n*-C27. On the other hand, the ratios of longer-chain alkanes, *n*-C27 and *n*-C29, was higher in the sexual generation without ant attendance. In this species, sexual females do not have organs that secrete sex pheromones to attract males. Thus, it is likely that the hydrocarbon components of the female body surface play a major role in mate recognition by males. The proportion of branched alkanes in the long and flocculent waxy substances of the winged autumn morphs was higher than in other morphs, indicating that this component may be used for recognition of the same species. These results indicate that the profiles and function of cuticular hydrocarbons differ among morphs, and that the cuticular hydrocarbons of sexual females in particular have differed significantly from those of asexually reproducing females and males for mate recognition as sexual attractants.

In Chapter 3, parasites of the Eriosomatinae were reported, and mermithid nematodes (Nematoda: Mermithidae) were found in the abdomens of aphids sucking on the secondary hosts underground. It is the first time found in Eriosomatinae that the root-generation aphids are parasitized by nematodes. I extracted genome DNA from the isolated nematode from *E. harunire*, sequenced the 18S rDNA and 28S rDNA regions, and performed phylogenetic analysis.

On October 9, 2017, in Yoichi, Hokkaido, Japan (43°12′9″ N, 140°45′52″ E), autumnal winged females (sexuparae) were collected using forceps just after their alighting on the branches of *Ulmus davidiana* and maintained in 80% ethanol. Sexuparae were dissected and slide-mounted with their embryonic sexual offspring in Hoyer's mountant for morphological observation (Tong & Akimoto 2019). When a parasite was found inside the sexuparae, it was isolated for later morphological and molecular identification. Aphids were identified morphologically, and all specimens were deposited in the Laboratory of Systematic Entomology, Hokkaido University, Sapporo, Japan.

One parasite found in an *Eriosoma auratum* sexupara was isolated, and its genomic DNA was extracted and purified using the DNeasy Blood and Tissue Kit (QIAGEN, Venlo, the Netherlands). The 18S ribosomal gene and the gene fragment of the large ribosomal subunit (LSU) 28S rDNA sequence were amplified and polymerase chain reaction (PCR) was performed according to Kobylinski *et al.* (2012) and Shih *et al.* (2019). The following primers were used: 18S, 18S-F: 5′- CAAGGAC GAAAGTTAGAGGTTC-3′ and 18S-R: 5′-GG AAACCTTGTTACGACTTTTA-3′, and

for 28S, LSU-F: 50–ACAAGTACCGTGAGGGAAAGTTG–30 and LSU-R: 50–TCGGAAGGAACCAGCTACTA–30 (Shih *et al.* 2019). The resulting templates were purified using a QIAquick PCR purification kit (QIAGEN Inc.) and sequenced in both directions using an ABI 3730xl Analyzer (Applied Biosystems). The resulting sequences were deposited in GenBank, and the BLASTn algorithm (Altschul *et al.* 1990) was applied to confirm the identity of the sequences.

The dataset of partial sequences of the nuclear 18S rDNA of mermithid nematodes in GenBank was searched and aligned using the MEGA X software package (Kumar *et al.* 2018). Host species were referenced to related publications and GenBank after obtaining 18S rDNA sequences of the parasitic mermithid nematodes (Table S1). Phylogenetic trees were constructed using Bayesian inference (BI) (Larget & Simon 1999) and maximum likelihood (ML) (Felsenstein 1981). The best-fit evolutionary model K2 + G + I was adopted by Mega X and used for all model-based methods (BI and ML). The Bayesian tree was constructed by MrBayes 3.2.7 (Ronquist et al. 2012) using a Markov chain Monte Carlo (MCMC) approach with 2 million generations, with tree sampling every 500 generations. The 1000 replicates were run for maximum likelihood (ML) bootstrap sampling using Mega X.

For aphids and other herbivorous hemipteran insects that share a common arrangement of sucking mouthparts, mermithid nematodes cannot enter host bodies through mouthparts. In the present study, the unidentified mermithid nematode likely parasitized the aphid by penetrating the cuticle, or gaining entry through a natural opening such as the trachea and anus, or during embryonic development being parasitized through maternal transmission.

Mermithid parasitism of aphids is not commonly known and only three cases have been reported (Guercio 1899; Davis 1916; Poinar 2017), although this could be due to undersampling of the aphids for this condition. The most remarkable record is the parasitism of an extinct aphid, *Caulinus burmitis* (Hemiptera: Burmitaphididae) by a fossil mermithid, which was found in mid-Cretaceous Myanmar amber (Poinar 2017). This example implies that the parasitic association between aphids and mermithid nematodes has continued for more than 100-million years. In Italy, nymphs and winged adults of the root aphid *Trama radices Kaltenbach* were found to be parasitized by an unidentified mermithid in April and May 1899, which was dispersed and embedded in the winged aphid (Guercio 1899). Davis (1916) conducted fieldwork to collect mermithid-parasitized aphids in Indiana, USA between mid-September and October 1911, and found mermithid-parasitized apterous viviparous and oviparous aphids of an *Anoecia* sp. on October 16th and 19th on the roots of *Muhlenbergia*. This is also the first record of mermithid parasitism in oviparous aphids.

The unidentified mermithid found in the present study was closest to a species collected from grassland soil around the root system of Leadplant, Amorpha canescens Pursh in the USA (Powers, pers. comm., also described in

https://nematode.unl.edu/mermissp.htm), which possibly contained herbivorous insects, including aphids. However, the taxonomic status of the nematodes is unknown in both cases since the samples were juveniles not closely aligned to any identified species. In addition, although the two species formed a well-supported clade in the phylogenetic analysis (Fig. 2), they were clearly separated from each other considering the branch length between them. Therefore, they possibly represent separate, undersampled clades. Further collections followed by phylogenetic analyses are required to understand their relationships and taxonomic status.

The survival and performance of parasites can be largely affected by their hosts. Nematodes receive nutrition from the host tissues and hemolymph, competing with the host for nutrients that are important for its physiological development and reproduction (Smith *et al.* 1985; Mcrae *et al.* 2015). Once mermithid nematodes parasitize host insects, they can manipulate host behavior for their own benefits. For example, Allahverdipour *et al.* (2019) reported that mermithid-parasitized female mosquitoes seek water three times more than a blood source, whereas uninfected females were twice as likely to seek blood than water. Moreover, parasitizing adult hosts could be a dispersal strategy for mermithid nematodes (Campos & Sy 2003; Di Battista *et al.* 2015). In the present study, obvious morphological or behavioral alterations were not confirmed in parasitized aphids and parasitism was not detected until dissection. Nevertheless, our study indicated that mermithid parasitism in sexuparae led to fewer and smaller female sexual embryos. It is not clear whether the parasites negatively affect offspring fitness by competing for nutritious resources directly or whether maternal investment changes in response to parasitism. Thus, it is necessary to increase the sample size to investigate host manipulation by mermithid nematodes in future studies.

Mermithid nematodes can infect a broad range of aquatic and terrestrial invertebrates. However, because nematodes are often collected as juveniles, their identification and host specificity are difficult to evaluate. *Mermis nigrescens*, a parasite of grasshoppers, is reported to be found in other insect orders, such as Dermaptera, Coleoptera, and Lepidoptera (Poinar 1979). However, because of the difficulty in morphological identification, information on the host range needs to be confirmed by molecular barcoding analyses. In the present study, although the species status is still unknown, the molecular sequences can be regarded as a species- specific barcode for taxonomic identification and evaluation of the host range in future studies.

In Chapter 4, I tested a hypothesis that sex investment ratio will always be biased toward females in fatal-fighting aphids with cyclic parthenogenesis. In general organisms, the investment in males and females is stable at 1:1, but in animals with cyclic parthenogenesis (e.g., rotifers, daphnia, aphids), it has been reported that the sexual investment ratio is often biased toward females. Using gall-forming aphids *T. sorini*, I tested the Trivers-Willard hypothesis and proposed a novel hypothesis. The first instar

nymphs hatching from overwintering eggs are all females, and fight fiercely for possession of the gall. The larger nymphs had an overwhelming advantage in the fight. In contrast, males rarely fought. Under these conditions, mothers were expected to have advantages if they produced more females of larger size with a sex investment ratio biased toward females. This is because larger daughters would produce larger granddaughters to be born in the spring.

In mid-October, autumnal winged females (hereafter "mothers") were collected using forceps just after alighting on the branches of *U. davidiana* and preserved in 80% ethanol. Two samples of mothers were used for analyses: (a) the "2001 sample" was collected in Iwamizawa, Hokkaido, Japan (43°11′28″N, 141°46′ 53″E), on 8 October 2001; and (b) the "2017 sample" was collected in Yoichi, Hokkaido, Japan (43°12′9″N, 140°45′52″E), on 9 October 2017. For the 2001 sample, all winged mothers were dissected and the numbers of males and females in the brood were recorded. The wing lengths of all mothers were measured after the forewings were slide-mounted using Hoyer's mountant. The forewing length was correlated with the length of hind femur ($r = 0.84$, $n = 20$, $p < 0.0001$) and was used as an index of body size. For the 2017 sample, all males and females in a brood and the forewings of the mother were mounted on a slide glass. All images were captured in a computer through a microscope eyepiece camera (Dino-Eye, AnMo Electronics Corporation, Taipei), and male and female body areas and forewing lengths were measured by using ImageJ software (http://rsbweb.nih.gov/ij/). To investigate the sex allocation patterns of *T. sorini* clones, we reared clones individually and obtained winged females from each clone. *Miscanthus sinensis* plants were collected from the wild in mid-May and transplanted into pots. Mature galls of *T. sorini* were collected from *U. davidiana* leaves in Iwamizawa, Hokkaido, Japan, in mid-June and placed in small plastic cages (30 mm width × 10 mm height × 30 mm depth) lined with dampened filter paper. After winged females (emigrants) appeared from the cleaved galls and parthenogenetically produced third-generation larvae, we transferred the larvae to the potted *M. sinensis* plants. Larvae from one gall were transferred to a single plant, and the plants were netted to prevent the entrance of different clones and predators. In mid-October, all winged mothers that emerged from the roots were collected. We used only 9 of the 10 clonal colonies for analyses because one of the clones produced fewer than 20 winged mothers. The other 9 clones produced from 21 to 120 mothers. All the mothers were dissected and the numbers of males and females in a brood and the wing length of the mother were recorded.

The cost of producing females was evaluated as described in Akimoto *et al.* (2012). This method postulates that mothers with the same wing length have the same amount of reproductive resources. We chose two groups of mothers: those that produced all females (abbreviated as all-F mothers), and those that produced six males and any number of females (a mother produces a maximum of six males). The number of females was compared between the two groups of mothers. Six-male-producing mothers produced

fewer females than all-F mothers (see Section 3). The difference in female numbers (i.e., K females) was attributed to the cost of producing six males. Therefore, if mothers are equivalent in body size, then the production cost of one female could be concluded to be equal to 6/K times the production cost of one male. Because the wing length varies among mothers, the number of females produced needed to be adjusted for this. We conducted regression analyses of the relationship between maternal wing length and the number of females produced for the two mother groups and then assessed whether regression lines with the same slope could be applied to the two mother groups using ANCOVA. In the model, the maternal wing length, mother groups and their interaction were treated as explanatory variables. If the interaction was not statistically significant, then regression lines with the same slope could be applied to both mother groups. In this case, the difference between the intercepts of the regression lines (K) was calculated using the least-square means. The 2001 and 2017 samples were used for computing the relative production cost of a female (F = 6/K). Investment in females was estimated to be (female number * F). The total maternal investment was calculated as (male number + female number * F). The relationship between the investment in females and the total maternal investment was analyzed by a simple linear regression.

In *T. sorini*, mothers invested to individual females 3.0-3.2 times larger than to individual males, and the ratio of sexual investment in females was 68%-72% at the population level. I confirmed that mothers with larger body size tended to produce more large females to have more fitness return.

The Trivers–Willard hypothesis predicts that mothers in better condition should bias the progeny sex ratios more towards males and invest more in individual males if they yield an increasing rate of reproductive returns on investment, but if females have a constant rate of reproductive returns (Frank 1987; Veller *et al.* 2016). This hypothesis has mainly been applied to mammals and birds (Clutton-Brock 1986; Clutton-Brock & Lason 1986; Cockburn *et al.* 2002; Frank 1990; Krackow 1995); however, it can also be applied to *T. sorini*, although the sex roles are reversed. Larger foundresses have an advantage in fighting, and they hatch from larger eggs. In eriosomatine aphids, the female lays only one egg, which is almost as long as herself (Heie 1980). Therefore, high maternal investment in individual females can consequently produce large foundresses (granddaughters). Our analyses of *T. sorini* mothers supported the Trivers–Willard hypothesis, indicating that more fecund mothers invested more in females in total; that is, they produced more female-biased broods and larger females. However, there was no tendency for more fecund mothers to produce larger males. In *T. sorini*, we confirmed that both of the predictions from the Trivers–Willard hypothesis are true, though the sex roles are reversed. Therefore, our result suggests that given additional maternal investment in offspring, increase in female fitness is larger than increase in male fitness if the offspring have received large investment, but smaller if they have received small resources. This prediction is consistent with our observation that larger foundresses, which are produced

by larger sexual females, have greater advantages in fighting, whereas males do not fight intensively. In conclusion, the pattern of maternal investment in *T. sorini* was consistent with the predictions from the Trivers–Willard hypothesis.

Theoretical studies have pointed out that predicting the population-level sex allocation is not straightforward because it varies depending on (a) the shapes of return curves for sons and daughters, (b) the distribution of maternal resources, and (c) trade-offs between the numbers and sizes of sons and daughters (Frank 1987, 1990; Frank & Swingland 1988; West 2009; Wild & West 2007). If return curves of sons differ from those of daughters, population sex ratios are predicted to be biased towards the sex produced by parents with relatively low levels of resources, but the population sex allocation ratio may be biased towards either sex (Frank 1990). These predictions were corroborated by *T. sorini*, which showed a male-biased sex ratio but a female-biased sex allocation at the population level. Provided that returns from investment in females increase linearly and maternal resources vary, population sex allocation is predicted to be biased towards females if returns from investment in males can be described by a positive exponential curve (Frank 1987, Figures 2 and 4, $t = 1$, $s > 1$) or an S-shaped curve ($t = 2$, $s \geq 3$). Since the sex roles are reversed in *T. sorini*, this theory predicts a larger allocation to males at the population level. Although the above-mentioned models evaluate the total reproductive returns on total male or female investment per investment period (e.g., one offspring at a time), an increasing number of offspring per period make the return curves more linear, bringing the predicted allocation closer to Fisher's equal allocation theory (Frank 1987, 1990). Therefore, theories predict male-biased or approximately equal population allocation for the case of *T. sorini*. However, our observation (an allocation of 68%–72% to females) is contradictory to this prediction.

Foundresses are adapted for fighting in their morphological specializations, including large body size, strongly sclerotized dorsum and stout elongated hind legs. If fighting occurs among relatives, from the viewpoint of inclusive fitness one would not expect the evolution of such a lethal fighting (but see West *et al.* 2001). The third factor is competition between unrelated foundresses. Several foundresses start galling on the same leaf and foundresses who failed to induce their own galls attack those who are inducing an incipient gall (Akimoto & Yamaguchi 1997). Muramatsu and Akimoto (2016) examined the body sizes of foundresses from nine populations, and in all of these, they detected significant directional selection for larger body size. Mothers who produce a larger number of bigger females could have more numerous granddaughters that survive the galling process and reproduce because of their better fighting ability. Thus, mothers' genetic returns would increase rapidly as they invest more in individual females. Of the three factors described above, competition among unrelated foundresses appears to have had the most significant effect on the evolution of female-biased sex allocation in *T. sorini* because its effects are stable and accompanied by the evolution of weapon morphology in foundresses. Conversely, LMC would occur only in very limited conditions such as those

of extremely low density, since males can disperse freely. LRC would also occur rarely and mainly result in male-biased population sex allocation. Our analyses indicated that mothers have the potential to control the sizes of female and male embryos. Mothers producing a few males produced larger-sized females than those producing four to six males. In addition, when mothers produced more females, males of the same brood were smaller in size. Therefore, complex trade-offs existed among the sizes and numbers of females and males. These trade-offs would have led to a dichotomy in brood sex composition to maximize maternal returns. In particular, to produce larger foundresses, mothers have to reduce the production of males, thus resulting in a high proportion of all-F mothers (28% in 2001 and 42% in 2017). In contrast, mothers who produce several males can obtain high returns through their sons' reproductive success. Therefore, these trade-offs between the need for specializations for female fighting ability or male function could explain the observed dichotomy in brood sex composition. In contrast, LMC models do not predict the occurrence of all-F mothers (Stubblefield & Seger 1990; Yamaguchi 1985) or variation in female size associated with the maternal nutritive status. Consequently, we postulate that competition among foundresses could have led to variation in maternal investment in individual daughters, as predicted by the Trivers–Willard hypothesis.