Host – parasitoid interaction in *Drosophila – Leptopilina* system

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Contents

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General Introduction

Parasitoids are defined as the organisms that develop as parasites of a single 'host' organism of other species, and kill the host as a direct or indirect result of that development (Kuris 1974; Eggleton and Gaston 1990). The term '*parasitoid*' was first coined by Reuter in 1913 to name the insects having such life style. Parasitoid insects occur in several orders of insect, e.g., Diptera, Coleoptera, Lepidoptera, Trichoptera, Neuroptera, Strepsiptera, but they are especially common in Hymenoptera where more than 75% of known species (50,000 species) are parasitoids (Gaston 1991; LaSalle and Gauld 1991; Godfray 1997; Quicke 1997; Whitfield 2003). The number of species of hymenopteran parasitoid wasps is estimated to constitute 10-20% of all insect species (Gaston 1991; LaSalle and Gauld 1991), consequently they are one of the most highly diversified and abundant groups of insects and important components in terrestrial ecosystem. In general, adults of parasitoid wasps (parasitoids) are free-living and females attack other insect species at immature stage, e.g., eggs, larvae or pupae, for oviposition. Larvae of parasitoids exploit nutrients for development from their only single host, and kill their host without allowing reproduction except a few species attacking adult insects (Askew 1971). Therefore, parasitoids can have strong impact on the fitness of their host and consequently selection pressures arisen from them influence host abundance and host life histories evolution. In contrasts, because parasitoids depend all the developmental resource for host individuals, abundance and life histories of hosts also influence on the parasitoids fitness vice versa. The fact that parasitoids and their hosts are strongly influence reciprocally makes the host-parasitoid system particularly suited to analyze various fundamental ecological and evolutionary processes driven by interaction between

antagonistic species with regard to life histories evolution, local adaptation, coevolutionary arms race, population and community dynamics, and so on (Boulétreau, 1986; Godfray and Shimada 1999; Hassel and Waage 1984; Jessup and Forde 2008; Rosenheim 1998).

One of the important factors characterizing the host-parasitoid interaction is the feeding behavior of parasitoid larvae and parasitoids are divided by it into either '*ectoparsitoids*' and '*endoparasitoids*'. Larvae of ectoparasitoids live and exploit externally, normally with their mouthparts buried in the body of their hosts. On the other hand, larvae of endoparasitoids live in the host body and feed their hosts from the inside. Endoparasitoids evolved from ectoparasitoids independently for multiple times in apocritan Hymenoptera, and this shift increased risks progeny facing the host's cellular immune response to cope with pathogens and parasites referred to as encapsulation. In this immune response, some hemocytes capsule around the parasitoid egg or larva resulting in death of parasitoid (Nappi 1975; Rizki and Rizki1984a; Nappi and Carton 1986). To defeat such host immune system, endoparasitoids evolve '*virulence*', the ability to avoid being detected by host immune systems or suppress host immune responses, which usually depends on the venom or virus injected by female wasp at oviposition (Edson et al. 1981; Shelby and Webb 1999; Eleftherianos et al. 2007). In contrast, evolution of parasitoid virulence increase host mortality, selection may drive the host adaptations to survive parasitoid attack by intensifying their immune responses or modifying their immune systems referred as to '*resistance*' (Strand and Pech 1995; Carton et al. 2008). Therefore, in host–endoparasitoid interaction, reciprocal selection arisen from virulence and resistance may result in the co-evolutionary arms race between hosts and parasitoids. Whether the host-parasitoid interaction resulting in co-evolutionary arms

race occurs is largely depending on the fitness cost or trade-offs associated with resistance, and genetic control of resistance and virulence (Doebeli 1997; Sasaki and Godfray 1999). *Drosophila* and its Hymenopteran larval-pupal parasitoids are the study model of such coevolution. Especially in *Drosophila melanogaster*, mechanisms, genetic control and cost of resistance were exceptionally examined. Once they are parasitized, some hemocytes change their shape and became very flattened (lamellocytes). Lamellocytes form the capsule around the parasitoid egg or larva which is rapidly melanized, a process that involves disintegration of another type of hemocyte, the crystal cell or oenocytoid (Nappi 1975; Rizki and Rizki1984a; Nappi and Carton 1986). Substantial additive genetic variation in host resistance has been found on a number of occasions (reviewed by Kraaijeveld et al. 1998) and many studies suggested that a few genes can switch either resistance against parasitoids although many genes involved in this immune process (Carton et al 1992; Dupas et al 1998; 2003; 2009; Hirata 2006; Dupbuffet et al. 2007; 2009). The cost of resistance was examined only in *D. melanogaster* so far. Kraaijeveld & Godfray (1999) and Fellows et al (1998) examined the fitness cost of evolution in resistance to *Asobara tabida* and *Leptopilina boulardi* in *D. melanogaster* based on the artificial selection experiment. They revealed that resistance against both parasitoid under trade-offs with intraspecific competitive ability under food limited condition, whereas under no trade-offs with other primal fitness related traits (e.g. larval and pupal survival, larval and pupal development time, adult longevity in the absence of food, early female fecundity). On the other hand, Rolff and Kraaijeveld (2003) found the higher mating success in selected males, suggesting an improvement of at least one aspect of fitness. In addition, it was also revealed in the study of Kraaijeveld and Godfray (1999) that the populations selected for resistance against an *A. tabida* population are also resistant to

conspecific parasitoid populations from different geographic regions, whereas show no increase in resistance to *L. boulardi*. On the other hand, although the populations selected for resistance against *L. boulardi* show some increase in resistance against *A. tabida* compared with the control populations (Fellowes et al. 1999). Thus, the resistance mechanism of *D. melanogaster* has parasitoid-species- or parasitoid-population-specific components. However, knowledge about parasitoid resistance in other *Drosophila* species are less understood. Some studies suggested that melanotic encapsulation is only common among *D. melanogaster* species subgroup, but other species frequently resists against parasitoids without melanization (Schlenke et al 2007; Salazar-Jaramillo et al 2014; Márkus et al 2015). In such species, cost and genetic control of resistance may differ with *D. melanogaster*, and they will provide important insight into the coevolutionary arms race between host and parasitoid.

In host-parasitoid system, physiological traits evolution of host insects other than immune system also influence on the fitness of parasitoids and evolution of life histories of parasitoids. For example, host size that parasitoid consumed critically influences on the parasitoids body size and development time (Harvey 2005). In many organisms, body size is considered as the primal target of fecundity selection (Jones 1982; King 1987; Shine 1988; Van den Assem et al. 1989; Heinz 1991). In insects, female enjoy more fitness gain by increasing body size than male resulting in the sexual size dimorphisms (SSD) where females are larger than males. The proximal factors that generate SSD is the sex-dependent extension of developmental period (i.e., sexual dimorphisms in development time or SDTD) in order to acquire more resource or sex-dependent variation in growth rate (Slansky 1993). SSD and SDTD are also common in parasitoid wasps. Generally, larvae of parasitoid wasps are totally dependent on a host insect for the nutrition necessary

to complete their development, and whether the host insect continues to grow after the infection may be an important factor that determines the host-wasp interaction. In the case of idiobiont parasitoids (idiobionts) that utilize non-growing hosts as their resource (Askew and Shaw 1986; Vinson 1988), host selection by adult female wasps plays an important role to generate SSD: ovipositing female eggs to large hosts and male eggs to small hosts (Schmidt and Smith 1985; Waage and Godfray 1985; Takagi 1986; Strand 1988; Hardy et al. 1992). So far, although SSD and SDTD were extensively studied in idiobionts, little is known about SSD and SDTD in koinobiont parasitoids (koinobionts) that utilize growing host insect as their resource. Because hosts infected by koinobionts often continue to develop and grow (Askew and Shaw 1986; Vinson 1988), developmental period and final body host size of the host insects at the time when it is consumed by the wasps and host developmental period reached to the size are expected to be a crucial factor that regulate wasp development (Harvey 2005), suggesting that they are also major determinant factors of SSD and SDTD.

In this thesis, I focused on the evolutionary aspect of physiological interactions between frugivorous *Drosophila* and their larval-pupal endoparasitoids of genus *Leptopilina*. There were two major aims in this study, (1) to reveal the cost and genetic control of resistance in *Drosophila* speices who resists without melanotic encapsulation and (2) to reveal how host potential growth differentiation influence on the koinobiont development in aspect of sexual dimorphisms in size and development time. ‘Chapter I’ aimed to reveal the evolutionary cost of resistance in *Drosophila bipectinata*. The previous knowledge on evolutionary cost of resistance is limited in *D. melanogaster* which from artificial selection based on the within population variation in resistance. In this study, I focused on the extensive geographic variation of resistance in *D. bipectinata*

against a *Leptopilina vicoriae* population. Such geographic variation may not be a simple extension of within population variation, but may differ in the kind or function of responsible genes. In addition, resistance in *D. bipectinata* does not involve melanization process suggesting that different resistance mechanisms with *D. malanogaster*. Three geographic population of *D. bipectinata* (two populations are resistant, but one is the susceptible to the *L. victoriae* population) were mixed for making base population and artificial selection experiment for improvement of resistance against the parasitoid. After that, I compared various life-history traits and resistance against other population or species of parasitoid among experimental (selected and control) and geographical populations to estimate the potential evolutionary cost of resistance against a parasitoid. 'Chapter II' aimed to reveal the how many genes associated with resistance differentiation between populations, and reveal genetic change in experimental populations during selection experiment in chapter I. In this chapter, I conducted the cross experiment between resistant and susceptible geographic population of *D. bipectinata* and amplified fragment length polymorphisms analysis for experimental and geographical population and experimental (selected and control) population used in selection experiment in Chapter I. 'Chapter III' aimed to reveal the influence of host potential growth differentiation on koinobiont development and their sexual dimorphisms. I focused on the influence of host SSD which common among *Drosophila* species on the koinobiont development. I examined development of each sex of *D. albomicans* under infection of either female or male of *L. ryukyuensis* and its effect of SSD and SDTD of *L. ryukyuensis*. In addition, I also examined whether the wasp allocate offspring sex depending on host sex that may maximize its fitness if host sex differently influences on wasp development.

Furthermore, host feeding rate were compared to examine whether the behavioral alteration account the host growth under infection.

Chapter I

Assessment of fitness costs of resistance against the parasitoid *Leptopilina vectoriae* in *Drosophila bipectinata*

I-1. Introduction

All insects have immune systems to defend themselves from infection of pathogens or parasites. However, their immune systems are not always effective, because some pathogens and parasites have means to avoid being detected by the host immune systems or suppress host immune responses (Edson et al. 1981; Shelby and Webb 1999; Eleftherianos et al. 2007). To cope with such enemy's adaptations, host insects often intensify their immune responses or modify their immune systems (Strand and Pech 1995; Carton et al. 2008). One of the important factors that affect such parasitoid-host coevolution is the costs of resistance and counter-resistance (Doebeli 1997; Sasaki and Godfray 1999). A powerful tool to examine these costs is the study of correlated responses to artificial selection. Kraaijeveld and Godfray (1997) and Fellowes et al. (1998) selected *Drosophila melanogaster* Meigen for improved resistance against *Asobara tabida* (Nees von Esenbeck) and *Leptopilina boulardi* (Barbotin, Carton and Kelner-Pillault), and found that the selected populations were inferior in competitive ability (survival under severe intraspecific competition) than the control populations. Fellowes et al. (1999) further indicated that lower competitive ability of the selected populations was associated

with reduced rates of larval feeding. In addition, Kraaijeveld et al. (2001) found that the selected populations have approximately twice the density of haemocytes than the control populations. On the other hand, males of the selected populations achieve a higher mating success than those of control populations (Rolff and Kraaijeveld 2003), suggesting an improvement of at least one aspect of fitness in the selected populations.

It has also been revealed in the study of Kraaijeveld and Godfray (1999) that the populations selected for resistance against an *A. tabida* population are also resistant to conspecific parasitoid populations from different geographic regions. However, fly populations resistant to a parasitoid population are not always resistant to other conspecific parasitoid populations. For example, some geographic populations of *D. melanogaster* from Africa show different responses to different geographic populations of *L. boulardi* (Dubuffet et al. 2007). In addition, the populations selected for resistance against *A. tabida* show no increase in resistance to *L. boulardi*, although the populations selected for resistance against *L. boulardi* show some increase in resistance against *A. tabida* compared with the control populations (Fellowes et al. 1999). Thus, the resistance mechanism of *D. melanogaster* has parasitoid-species- or parasitoid-population-specific components.

The above selection studies were based on within-population genetic variation. Resistance and counter-resistance against antagonists often show more extensive variation geographically (Carton et al. 1992; Kraaijeveld and van Alphen 1994; Dupas et al. 1998; Hufbauer 2001). For example, an African population of *D. melanogaster* has complete resistance against a population of the parasitoid *L. boulardi*, whereas another population has no resistance against the same parasitoid population (Dupas et al. 1998). Such geographic variation may not be a simple extension of within-population variation,

but may differ in the kind or function of responsible genes. However, there has been no selection study based on geographic variation.

In this study, I assessed fitness costs or trade-offs associated with resistance of *Drosophila bipectinata* Duda against the parasitoid *Leptopilina victoriae* Nordlander using host populations from different geographic regions. *D. bipectinata* occurs throughout Southeast Asia, extending into South Pacific islands, Australia, India and Africa (Bock and Wheeler 1972; Lemeunier et al. 1986; Kopp and Barmia 2005). Novković et al. (2012) reported that a *D. bipectinata* population from Iriomote-jima (southernmost Japan) is susceptible to *L. victoriae* from Kota Kinabalu (Malaysia), but its populations from Kota Kinabalu and Bogor (Indonesia) are resistant to this parasitoid population. Our preliminary study suggests that resistant populations of *D. bipectinata* show no melanization against *L. victoriae* eggs or embryos (Takigahira, unpublished data), suggesting that this species has a different resistance mechanism from *D. melanogaster* that shows a melanization response to parasitoids.

Iproduced a base population of *D. bipectinata* by mixing geographic populations from Iriomote-jima, Kota Kinabalu and Bogor, and selected for resistance against *L. victoriae* from Kota Kinabalu. To assess fitness costs that are associated with the resistance, I compared life history and stress tolerance traits widely between the selected and control populations, because it cannot be predicted what traits will exhibit trade-offs with parasitoid resistance. We also compared resistance/susceptibility against another population of *L. victoriae* and two other parasitoid species whether the resistance is under trade-off with resistance against other parasitoid species or population.

I-2. Materials and methods

I-2-1. Selection for parasitoid resistance

The base population for the selection experiments was produced by mixing three populations of *Drosophila bipectinata* derived from females collected from Bogor (BG, Indonesia: 6.6 °S, 106.8 °E) in June 2008, Kota Kinabalu (KK, Malaysia: 5.3 °N, 117.4 °E) in March 2008, and Iriomote-jima (IR, Japan: 24.4 °N, 123.9 °E) in March 2005. The *D. bipectinata* populations from BG and KK (*D. bipectinata* BG and KK) are resistant to *L. victoriae* KK, while *D. bipectinata* IR is susceptible (Novković et al. 2012). These populations were maintained on Drosophila medium under 15L: 9D (15 h light: 9 h dark) at 23°C in laboratory for a few years. To establish the base population, 20 females and 20 males from each population were mixed and maintained with free mating for six generations before starting the selection experiments.

The base population of *D. bipectinata* was divided into four experimental populations, two for selection of resistance against *L. victoriae* KK and two for control. The *L. victoriae* KK population used for the selection experiments originated from females collected in Kota Kinabalu in March 2008 and maintained in mass culture (100 – 200 females in each generation) using *Drosophila simulans* Sturtevant (originated from Sapporo, Japan) as host.

Selection was performed as follows. One- to two-day old *D. bipectinata* larvae were placed in a Petri dish containing a small amount of rearing medium and then exposed to several female wasps, whose oviposition behavior was followed under a stereoscopic microscope. Characteristic oviposition behavior, such as full extension of the ovipositor

after contact with host and longer insertions of the ovipositor into larvae (>10 s) were taken as indicators of successful oviposition. When oviposition was confirmed, parasitized fly larvae were transferred into vials containing Drosophila medium. Thus, all larvae were subjected to single parasitism (parasitized by one parasitoid individual). One hundred parasitized larvae were prepared for each selected population and survivors were collected to produce the next generation. The number of survivors (i.e., the number of individuals to produce the next generation) was 20-25 in the first few generations of selection but soon increased over 40. Thus, the selected populations were maintained without selection after 10 generations of selection.

The control populations were maintained without parasitism treatment; i.e. 100 larvae were randomly chosen for each control population and flies that emerged were collected to produce the next generation. The control populations were also monitored for the resistance against *L. victoriae* KK every generation in the first 10 generations and at the 20th generation; 100 parasitized larvae were prepared for each control population and the number of flies and wasps that emerged were counted.

Female wasps used for selection and monitoring the control populations were always taken from the population of *L. victoriae* KK maintained using *D. simulans* as host; i.e., they had not experienced coevolutionary interactions with *D. bipectinata* at least in the laboratory. Usually more than 10 female wasps were used to prepare 100 parasitized *D. bipectinata* larvae to avoid a bias due to the individual variation of virulence in wasps. The resistance was determined by the following equation; resistance index = $F/(F+W)$, where F was the number of flies that emerged and W was the number of wasps that emerged. The experimental populations were also maintained under 15L: 9D at 23°C.

1-2-2. Measurements of life history and stress tolerance traits

To assess the cost associated with parasitoid resistance, unparasitized individuals of the selected and control populations were measured for the following life history and stress tolerance traits after eight or nine generations of experimental treatments (with/without selection). In addition, life history and stress tolerance traits of the three original populations were measured for reference. Except individuals examined for the competitive ability, those used for the measurements of life history and stress tolerance traits were reared at a low density (<50 larvae per 10 ml Drosophila medium) to minimize harmful effects of high density.

Egg-to-adult development time and viability

Adult flies were introduced into vials (50 ml) with Drosophila medium and allowed to oviposit for 6 hours. Eggs were collected, introduced into new vials (25 eggs per vial) with Drosophila medium (10 ml), and placed under a continuous light at 23°C. Flies that emerged from vials were counted every 6 hours. Five replicates were prepared for each population.

Longevity

Newly eclosed flies were transferred into new vials with Drosophila medium under a continuous light at 23°C. Flies were transferred into new vials every one or two day(s) and survivors were counted every day. Measurements were made with three replicates, each with 15~20 individuals of each sex from each population.

Female fecundity

One newly eclosed virgin female and two males were randomly paired and introduced into a vial (30 ml) with Drosophila medium (6 ml) under a continuous light at 23°C. Flies were transferred into a new vial every day, and eggs oviposited in the old vial were counted. Measurements were made for 20 days with 21 replicates for each population.

Thorax length

Adult flies were collected from vials used in the above "development time and viability" experiments, placed in vials with Drosophila medium for 2 or 3 days, and fixed in 70% ethanol. Thorax length was measured for approximately 30 individuals of each sex from each population.

Larval competitive ability

In their study using *D. melanogaster*, Kraaijeveld and Godfray (1997) assessed intraspecific competitive ability; i.e., competitive ability against a conspecific mutant strain. In the present study, however, competitive ability against a different species, *D. simulans*, was assessed, because an appropriate mutant strain was not available in *D. bipectinata*. Both of *D. simulans* and *D. bipectinata* are fruit-feeders mainly exploiting succulent fruits (Hirai et al. 2000; Mitsui and Kimura 2010; Novković et al. 2012) and are assumed to be competitive. Methods for measurement of larval competitive ability followed Kraaijeveld and Godfray (1997). The agar lined vials with 0.05 ml of yeast medium (25 g yeast per 100 ml water) were prepared. Twenty two-day old larvae of each experimental population were introduced into a vial with 20 two-day old *D. simulans* larvae as tester flies, and emergence of *D. bipectinata* and *D. simulans* was examined.

Measurements were made with 10 replicates for each population. The competition index was calculated by the following formula, log ((*b*+1)/(*t*+1)), where *b* is the number of flies of each population and *t* is the number of tester flies.

Viability at low and high temperatures

Egg-to-adult viability was examined at temperatures of 16 and 31.5°C in the same way as described above. These temperatures are close to lower and upper limits for the egg-to-adult development of *D. bipectinata*, respectively (see Results).

Cold and heat tolerance

Larval and adult survival was examined at low and high temperatures that occur in the habitats or distribution range of the study species. To examine larval tolerance, three-day-old larvae were introduced into new vials with Drosophila medium, exposed to 10.5 or 35.5°C for 24 h, and then placed at 23°C. The number of flies that emerged from these vials was examined. Measurements were made with two replicates, each with approximately 50 individuals from each population. To examine adult tolerance, 7 to10-day old adult flies were placed at 7.5 or 34°C for 24 h, then placed at 23°C for 24 h for recovery, and examined for survival. Flies that were able to walk were assigned as survivors. Measurements were made with two replicates, each with approximately 20 individuals for each sex from each population.

Starvation tolerance

Adult flies (7-10 days after eclosion) were introduced into vials with non-nutritional medium containing only agar and water and placed under a continuous light at 23°C.

Survivors were counted every 6 hours. Measurements were made with eight replicates, each with approximately 10 individuals for each sex from each population.

Desiccation tolerance

Adult flies (7-10 days after eclosion) were introduced into empty vials covered with nylon gauze and placed in a desiccator (25×25×37 cm) with fresh silica gel under a continuous light at 23°C. In the desiccator, humidity fell below 10% within 1 h and gradually decreased further. Survivors were counted every 30 min. Measurements were made with two replicates, each with approximately 20 individuals for each sex from each population.

I-2-3. Resistance against other parasitoids

Resistance/susceptibility of the original populations and the selected and control populations against *L. victoriae* BG, *L. ryukyuensis* Novković & Kimura IR and *Asobara pleuralis* (Ashmead) KK was examined by parasitism experiments. *L. victoriae* BG originated from several females collected from Bogor in June 2008; *L. ryukyuensis* IR from those collected from Iriomote-jima in March 2005; *A. pleuralis* KK from those collected from Kota Kinabalu in March 2008. These parasitoid populations were maintained under 15L: 9D (15 h light: 9 h dark) at 23°C in laboratory for a few years using *D. simulans* as host. For the selected and control populations, flies of the eighth or ninth generation of the selection/control treatments were used. Parasitized larvae were prepared as explained previously (50 larvae for each population or each population), and the number of emergent flies or wasps was examined.

I-2-4. Data analysis

Longevity, starvation and desiccation tolerance were analyzed by survival analysis using "survival" package (Therneau and Lumley 2014) in R software version 2.15.1 (R Development Core Team, 2012). All survival models include the populations as a predictor variable and statistical significance of the predictor variable was obtained using log-lank test. We conducted post-hoc multiple comparisons for the traits that were shown to have significant effect of predictor variable. Significance levels among populations were corrected by Holm's method (Holm 1979).

Other measured life history and stress tolerance traits were analyzed by fitting the generalized liner models (GLMs) using maximum likelihood in R. All GLMs include the populations as a predictor variable. To test statistical significance of the predictor variable, I calculated difference between -2 log likelihood of the model and null model using likelihood ratio test (LRT). For the traits that were shown to have significant effect of predictor variable, post-hoc multiple comparisons were conducted to assess the difference among the populations. Multiple comparisons among the populations of egg-to-adult development time and thorax length were carried out using the "multcomp" package (Hothorn et al. 2013) in R. Viability, heat and cold tolerance was analyzed by Fisher's exact test with correction of significance levels by Holm's method. The three original populations and the experimental (selected and control) populations were analyzed independently in all life history and stress tolerance traits.

Resistance against other parasitoid population or species was analyzed by fitting the GLMs, and differences among populations were analyzed using L**I-3. Results**

I-3-1. Response to selection

The resistance of the base population (Generation 0 in Fig. I-1) against *L. victoriae* KK was low (resistance index: 0.15). The selected populations rapidly increased the resistance, and the resistance index reached 0.80 within four generations in the two replicate populations (Fig. I-1). Resistance did not fall for at least 10 generations after selection was stopped at the 10th generation. In the two control populations, the resistance remained at low levels (resistance index: 0.1-0.3) except the second generation.

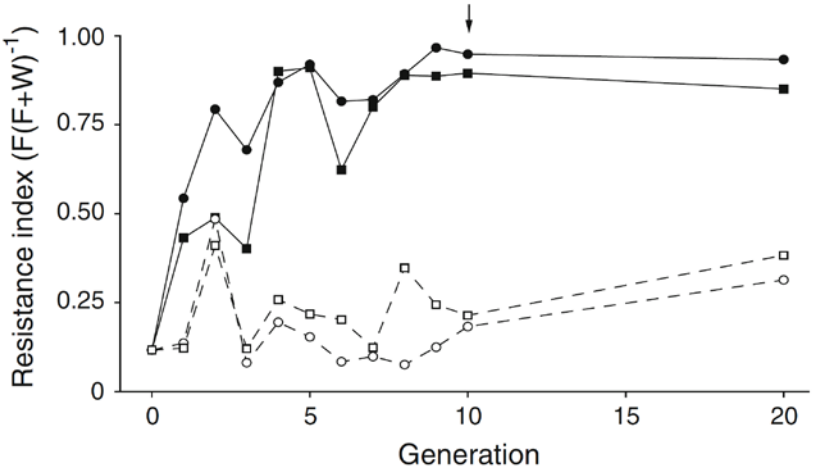


Fig. I-1 Response to selection. Control populations (C1; open circle, C2; open square) and selected populations (S1; closed circle, S2; closed square). Selected populations were maintained without selection after 10th generation (indicated by arrow). Resistance of each population was calculated from the numbers of flies (F) and wasps (W) that emerged from 100 fly larvae that were parasitized by wasps.

I-3-2. Life history and stress tolerance traits

Most life history and stress tolerance traits varied among the three original populations (Table I-1). Consistent significant differences between resistant (BG and KK) and susceptible (IR) populations were observed in egg-to-adult development time, female longevity, egg-to-adult viability at 16 °C, male heat tolerance, starvation tolerance and female desiccation tolerance.

Among the selected and control populations, significant differences were observed in 11 traits; i.e., female and male longevity, female thorax length, female and male starvation tolerance, female desiccation tolerance, female and male adult heat tolerance, larval survival at heat and egg-to-adult viability at 23 and 31.5 °C (Table I-2). Among these traits, only female longevity was lower in both selected populations compared with the two control populations, and thorax length was shorter and female desiccation tolerance and adult heat tolerance were lower at least in one of the selected populations compared with both or either of the control populations. In contrast, male longevity, male starvation tolerance and larval heat tolerance were higher in the selected populations compared with both or either of the control populations. Female starvation tolerance and egg-to-adult viability showed no distinct trend.

Table I-1. Mean ± SE (n) for life history and stress tolerance traits in three original populations (BG: Bogor, KK: Kota Kinabalu, IR: Iriomote-jima) and results of statistical analysis in three original populations. Different letters indicate statistically significant difference ($P < 0.05$) in post hoc multiple comparison.

Traits	Sex	Population			χ^2	*df*	*P* value
		BG	KK	IR			
Egg-to-adult development time (h)	Female	228.8 ± 1.12 (45)b	236.7 ± 1.36 (40)c	224.4 ± 0.48 (55)a	64.2	2	< 0.0001
	Male	233.7 ± 1.23 (61)b	238.9 ± 1.31 (37)c	228.7 ± 0.89 (54)a	31.4	2	< 0.0001
Egg-to-adult viability at 23.0 °C (%)	-	84.8 (125)b	61.6 (125)a	87.2 (125)b	27.8	2	< 0.0001
Longevity (day)	Female	36.4 ± 1.28 (90)a	35.3 ± 1.72 (79)a	40.8 ± 1.72 (111)b	12.8	2	0.0017
	Male	51.1 ± 1.82 (88)a	62.4 ± 2.56 (63)b	52.2 ± 1.49 (84)a	23.4	2	< 0.0001
Female fecundity (No. of egg)	-	293.6 ± 19.52 (21)	215.8 ± 32.59 (21)	247.0 ± 29.38 (21)	0.8	2	0.6782
Thorax length (μm)	Female	888.3 ± 4.52 (30)a	934.6 ± 4.86 (30)b	902.5 ± 4.17 (30)a	44.0	2	< 0.0001
	Male	773.3 ± 3.92 (30)a	795.4 ± 4.46 (30)b	776.3 ± 4.42 (30)a	15.0	2	0.0006
Larval competitive ability (Index)	-	−0.17 ± 0.060 (10*)	−0.38 ± 0.077 (10*)	−0.07 ± 0.023 (10*)	5.0	2	0.0831
Egg-to-adult viability at 16.0 °C (%)	-	0.0 (100) a	8.0 (100) b	22.0 (100) c	33.9	2	< 0.0001
Egg-to adult viability at 31.5 °C (%)	-	5.0 (100)	13.0 (100)	7.0 (100)	4.4	2	0.1112
Larval survival at 10.5 °C (%)	-	15.0 (100) b	2.0 (100) a	15.0 (100) b	15.0	2	0.0006
Larval survival at 35.5 °C (%)	-	48.0 (100) b	27.0 (100) a	50.0 (100) b	13.8	2	0.0010
Adult survival at 7.5 °C (%)	Female	80.8 (52) b	23.9 (43) a	20.9 (46) a	47.8	2	< 0.0001
	Male	65.9 (41) b	16.7 (42) a	2.7 (37) a	45.2	2	< 0.0001
Adult survival at 34.0 °C (%)	Female	93.8 (48) b	51.2 (41) a	69.4 (49) a	22.8	2	< 0.0001
	Male	89.5 (38) c	50.0 (36) b	22.7 (44) a	40.6	2	< 0.0001
Survival time under starvation (h)	Female	70.8 ± 1.87 (53)b	76.4 ± 2.21 (52)b	58.2 ± 1.55 (49)a	45.7	2	< 0.0001
	Male	62.6 ± 2.30 (28)c	58.1 ± 1.06 (50)b	31.9 ± 0.87 (37)a	116.0	2	< 0.0001
Survival time under desiccation (h)	Female	4.03 ± 0.113 (43)b	3.60 ± 0.118 (46)a	5.04 ± 0.129 (42)c	47.3	2	< 0.0001
	Male	3.69 ± 0.140 (42)	3.44 ± 0.152 (42)	3.32 ± 0.110 (43)	4.2	2	0.1230

*Number of vials. See method of larval competitive ability.

Table 1-2. Mean ± SE (n) for life history and stress tolerance traits in selected and control populations and results of statistical analysis. Different letters indicate statistically significant difference ($P < 0.05$) in post hoc multiple comparison.

Traits	Sex	Control populations		Selected populations		χ^2	*df*	*P* value
		C1	C2	S1	S2			
Egg-to-adult development time (h)	Female	223.2 ± 0.84 (50)	224.9 ± 0.70 (47)	224.9 ± 0.82 (49)	222.9 ± 0.78 (54)	5.9	3	0.1191
	Male	229.3 ± 1.07 (50)	228.4 ± 0.98 (34)	230.1 ± 1.09 (20)	230.4 ± 1.22 (37)	1.9	3	0.5944
Egg-to-adult viability at 23.0 ℃ (%)	-	80.0 (125) a	64.8 (125) bc	55.2 (125) c	72.8 (125) ab	19.8	3	0.0002
Longevity (day)	Female	51.0 ± 2.03 (107) a	45.7 ± 2.10 (90) ab	39.3 ± 3.08 (46) bc	37.9 ± 1.95 (73) c	22.6	3	< 0.0001
	Male	59.8 ± 2.40 (69) b	56.2 ± 2.61 (61) b	73.6 ± 2.69 (34) a	71.6 ± 2.19 (57) a	20.2	3	0.0002
Female fecundity (No. of egg)	-	360.9 ± 32.39 (21)	364.7 ± 21.36 (21)	336.1 ± 28.15 (21)	304.4 ± 23.50 (21)	2.2	3	0.5418
Thorax length (μm)	Female	924.2 ± 5.36 (30) b	937.1 ± 3.81 (30) ab	941.3 ± 4.24 (30) a	937.1 ± 4.50 (30) ab	8.1	3	0.0441
	Male	792.5 ± 3.21 (30)	797.1 ± 3.73 (30)	803.6 ± 5.06 (28)	793.8 ± 4.75 (30)	4.1	3	0.2517
Larval competitive ability (Index)	-	−0.24 ± 0.072 (10*)	−0.22 ± 0.031 (10*)	−0.35 ± 0.069 (10*)	−0.17 ± 0.056 (10*)	1.3	3	0.7339
Egg-to-adult viability at 16.0 ℃ (%)	-	18.0 (100)	20.0 (100)	14.0 (100)	17.0 (100)	1.3	3	0.7212
Egg-to adult viability at 31.5 ℃ (%)	-	9.0 (100) ab	22.0 (100) a	13.0 (100) b	26.0 (100) ab	13.2	3	0.0042
Larval survival at 10.5 ℃ (%)	-	20.0 (100)	22.0 (100)	19.0 (100)	15.0 (100)	1.7	3	0.6303
Larval survival at 35.5 ℃ (%)	-	61.0 (100) b	67.0 (100) a	34.0 (100) a	56.0 (100) a	25.3	3	< 0.0001
Adult survival at 7.5 ℃ (%)	Female	36.4 (44)	54.5 (44)	35.9 (39)	53.8 (39)	5.5	3	0.1380
	Male	6.7 (45)	10.5 (38)	0.0 (36)	8.3 (36)	5.9	3	0.1172
Adult survival at 34.0 ℃ (%)	Female	76.7 (43) a	66.7 (42) ab	22.9 (35) c	46.3 (41) bc	27.4	3	< 0.0001
	Male	40.0 (40) a	38.6 (44) ab	13.2 (38) b	29.5 (44) ab	9.2	3	0.0262
Survival time under starvation (h)	Female	57.9 ± 1.49 (80)ab	62.3 ± 1.33 (84)a	57.8 ± 1.17 (76)b	60.6 ± 1.31 (67)ab	7.9	3	0.0482
	Male	36.3 ± 0.77 (93) b	37.4 ± 0.93 (81) b	40.8 ± 0.91 (100) a	41.3 ± 0.77 (106) a	24.3	3	< 0.0001
Survival time under desiccation (h)	Female	5.38 ± 0.122 (40) a	5.33 ± 0.196 (39) ab	4.87 ± 0.123 (45) b	5.55 ± 0.187 (44) a	14.9	3	0.0019
	Male	3.59 ± 0.137 (43)	3.57 ± 0.110 (41)	3.61 ± 0.116 (43)	3.99 ± 0.149 (39)	5.8	3	0.1240

*Number of vials. See method of larval competitive ability.

I-3-3. Resistance against other parasitoids

All of the original geographic populations and the selected and control populations were highly resistant to *L. victoriae* BG, a less virulent population compared with *L. victoriae* KK that was used in the selection experiment (Table I-3). These populations also had resistance against *L. ryukyuensis* IR (Table I-3). On the other hand, resistance against *A. pleuralis* KK significantly varied among the populations (LRT: $\chi^2 = 57.3$, $df = 6$, $P < 0.001$): *D. bipectinata* BG was rather resistant and *D. bipectinata* KK was slightly resistant, while *D. bipectinata* IR and the selected and control populations were almost susceptible. Among the selected and control populations, no significant difference was observed in the resistance against *A. pleuralis* KK (LRT: $\chi^2 = 3.9$, $df = 3$, $P = 0.270$).

I-4. Discussion

The base population was constructed from two geographic (BG and KK) populations resistant against *L. victoriae* KK and one susceptible (IR) population. If each original population equally contributes to the genetic constitution of the base population, the base population would have a rather high resistance. However, it showed a relatively low resistance. The BG and KK populations may have possessed some low-fitness genes with which the resistance gene(s) are linked, and then the resistance may have been lowered in the base population before the linkage between these genes has been broken by recombination. Indeed, both or either of the BG and KK populations showed slower development, lower viability at 23 °C and shorter female longevity than the IR population, although male longevity was longer in the KK population compared with the IR

population and fecundity and larval competitive ability did not significantly differ among these three populations (Table 1).

The selected populations rapidly increased resistance and became highly resistant to *L. victoriae* KK within four generations. This may suggest that the number of genes responsible for the difference in resistance between the IR population and the BG or KK populations is few. Indeed, simple genetic control of parasitoid resistance has also

Table I-3. Results of parasitism by *Leptopilina victoriae* BG, *Leptopilina ryukyuensis* IR and *Asobara pleuralis* KK in the original (BG, KK and IR) populations and the selected (S1 and S2) and control populations (C1 and C2) of *D. bipectinata*.

	L. victoriae BG			*L. ryukyuensis* IR			*A. pleuralis* KK		
Population	F	W	D	F	W	D	F	W	D
BG	41	0	9	30	0	20	17	11	22
KK	39	0	11	38	0	12	5	31	14
IR	35	0	15	38	0	12	1	33	16
C1	45	0	15	30	0	20	3	34	13
C2	39	1	10	41	0	9	1	31	18
S1	37	0	13	39	0	11	1	39	10
S2	45	0	5	42	0	8	0	29	21

F: number of flies that emerged, W: number of wasps that emerged, D: number of host larvae from which neither fly nor wasp emerged. No significant difference was observed in resistance against the three parasitoid strains at least among the selected and control lines (likelihood ratio test, $P<0.05$).

been reported in *D. melanogaster* and *D. yakuba* Burla (Carton et al. 1992; Kraaijeveld and van Alphen 1995; Dupas et al. 1998, 2003, 2009; Dubuffet et al. 2007, 2009). population and fecundity and larval competitive ability did not significantly differ among these three populations (Table 1).

The selected populations rapidly increased resistance and became highly resistant to *L. victoriae* KK within four generations. This may suggest that the number of genes responsible for the difference in resistance between the IR population and the BG or KK populations is few. Indeed, simple genetic control of parasitoid resistance has also been reported in *D. melanogaster* and *D. yakuba* Burla (Carton et al. 1992; Kraaijeveld and van Alphen 1995; Dupas et al. 1998, 2003, 2009; Dubuffet et al. 2007, 2009).

In the present study, only female longevity was reduced in both selected (resistant) populations compared with the two control (susceptible) populations, but male longevity showed an opposite trend. On the other hand, female longevity did not significantly differ between the original resistant (BG and KK) and susceptible (IR) populations, and male longevity was low not only in the IR population but also in the BG population. Among the other traits, female desiccation tolerance and adult heat tolerance were reduced in one of the two selected populations. Female desiccation tolerance was also lower in the BG and KK populations compared with the IR population, but adult heat tolerance was much higher in the BG population compared with the KK and IR populations. Thus, the resistance against *L victoriae* KK may incur some costs to *D. bipectinata*, but it would not be high. This notion is supported by the present selection experiments where the resistance changed little for 10 or 20 generations in the selected and control populations if there was no artificial selection. However, it is still possible that the differences between the selected and control population is attributable to random drift, since the number of

individuals used to produce the next generation was not large (i.e., 20-25) in the first few generations of selection. In addition, there may be some costs that cannot be detected by such laboratory experiments.

In previous selection experiments using *D. melanogaster*, a trade-off was observed between larval competitive ability and resistance against *L. boulardi* and *A. tabida* (Kraaijeveld and Godfray 1997; Fellowes et al. 1998). In the present study, however, no significant difference was observed in competitive ability between the selected and control populations. This may be attributable to the difference in the drosophilid and parasitoid species studied or the difference in the type of competition; the previous studies examined intraspecific competition, whereas this study examined interspecific competition.

Irrespective of susceptibility/resistance against *L. victoriae* KK, all populations were resistant to *L. victoriae* BG and *L. ryukyuensis* IR, and all excepting the BG population were almost susceptible to *A. pleuralis*. Such parasitoid–species–specificity in resistance has been reported in a number of *Drosophila* species, and parasitoid–population–specificity has also been observed in some species (Dupas et al. 1998, 2003, 2009; Dubuffet et al. 2007, 2009; Mitsui and Kimura 2010; Novković et al. 2012; Kimura and Suwito 2014). If resistance is thus parasitoid–species–specific, host *Drosophila* species would be required to evolve a number of different resistance mechanisms, because they usually encounter a number of parasitoid species in nature (Mitsui and Kimura 2010; Kimura and Suwito 2012; Novković et al. 2012). Indeed, *D. bipectinata* from Iriomote-jima is resistant against *L. victoriae* BG, *L. ryukyuensis* and *Asobara japonica* Belokobylskij, and probably to *L. pacifica* Novković & Kimura (Novković et al. 2012). The low-cost nature of resistance may be important for host species to cope with a number

of different parasitoid species.

Drosophila bipectinata is widely distributed in tropical Asia, and Iriomote-jima is located near the northern boundary of its distribution (Bock and Wheeler 1972; Lemeunier et al. 1986; Kopp and Barmia 2005). This species is cold susceptible and its population in Iriomote-jima suffers high mortality in winter (Hirai et al. 2000; Kimura 2004; Novković et al. 2012). These things suggest that *D. bipectinata* has originated in the tropical regions of Asia and colonized Iriomote-jima rather recently. *Leptopilina victoriae* showed a similar distribution with *D. bipectinata*, but it occurs very rarely or only sporadically in Iriomote-jima (Nordlander 1980; Novković et al. 2011, 2012). It is therefore assumed that *D. bipectinata* in Iriomote-jima has lost resistance to *L. victoriae* KK, possibly as a result of low parasitism intensity (Novković et al. 2012). However, it is not known why *D. bipectinata* in Iriomote-jima still maintains resistance against *L. victoriae* from Bogor.

In conclusion, the resistance of *D. bipectinata* against *L. victoriae* KK probably incurs low fitness costs and specific to certain parasitoid populations or species (also see Dupas and Boscaro 1999; Kraaijeveld et al. 2001; Dupas et al. 2009; Mitsui and Kimura 2010; Novković et al. 2012). In general, *Drosophila*-parasitoid systems are multispecific, i.e., a host species is parasitized by more than one parasitoid species, and a parasitoid species parasitizes more than one host species (Dupas et al. 2009; Mitsui and Kimura 2010; Kimura and Suwito 2012; Novković et al. 2012). If virulence and resistance are specific to a certain antagonist, such multispecific systems are possible only when virulence and resistance incur low costs; if a resistance to a parasitoid species is costly, it would be difficult to acquire resistance against a number of parasitoids. For further understanding of parasitoid-host associations, thus, it is important to assess the cost and

specificity of virulence and resistance. One of important approaches to address this issue is identification of virulence and resistance genes by quantitative trait loci analysis using AFLP or next-generation sequencers.

Chapter II

Genetic analyses of resistance against *Leptopilina victoriae* in *Drosophila bipectinata*

II-1. Introduction

In host-parasitoid systems, hosts may evolve resistance against parasitoids if the parasitoid attack has serious effects on the host's populations, resulting in the evolution of parasitoid's counter-resistance (Godfray 1994; Hawkins 1994). To understand whether or not such coevolutionary arms race occurs, knowledge on the genetic control of resistance and counter-resistance is very important. Generally, host resistance relies on the immune system that works under a harmonic expression of a large number of genes (Hultmark 2003; Lemaitre and Hoffmann 2007), and parasitoid virulence relies on complex biochemical and physiological systems that suppress the host immune responses and modify the host conditions suitable for the development of parasitoid embryos (Vass and Nappi 2000; Moreau and Guillot 2005). However, the loss and gain of host resistance or parasitoid counter-resistance do not necessarily require extensive genetic changes but occur with simple genetic changes. For example, the cellular encapsulation responses of *Drosophila melanogaster* against *Leptopilina clavipes* eggs is controlled by some proteins in the N glycosylation pathway (Mortimer et al. 2012), and virulence/avirulence of a parasitoid *L. boulardi* against *D. melanogaster* was dependent on the expression level

of a immune suppressive protein in venom (Colinet et al. 2007, 2010). These proteins would play key roles in the resistance and virulence mechanisms.

In this chapter, I analyzed the genetic control of resistance against a parasitoid *Leptopilina victoriae* Nordlander in *Drosophila bipectinata* Duda. *Drosophila bipectinata* and *L. victoriae* occur throughout Southeast Asia, extending into South Pacific islands, Australia, India and/or Africa (Bock and Wheeler 1972; Lemeunier et al. 1986; Carton et al. 1986; Kopp and Barmia 2005). Novković et al. (2012) reported that a population of *D. bipectinata* from Iriomote-jima (IR: Japan) was susceptible to *L. victoriae* from Kota Kinabalu (*L. victoriae* KK), but those from Kota Kinabalu (KK: Malaysia) and Bogor (BG: Indonesia) were resistant to this parasitoid population. The number of genes causing this difference in resistance may be few, because an experimental population produced by mixing these IR, KK and BG populations rapidly responded artificial selection for increased resistance against *L. victoriae* KK in our previous study (Takigahira et al. 2014). Here, I verify this notion by cross experiments using the resistant KK and susceptible IR populations.

In addition, I conducted amplified fragment length polymorphism (AFLP) analysis to understand the genetic basis of resistance of *D. bipectinata* against *L. victoriae* KK. In Chapter I (Takigahira et al. 2014), a base population was established by mixing the IR, KK and BG populations of *D. bipectinata* and allowed to mate freely for six generations. At the sixth generation, the base population had low resistance against *L. victoriae* KK, although it was a mixture of one susceptible (IR) population and two resistant (KK and BG) populations with the same proportion (Takigahira et al. 2014). Thereafter, however, the resistance changed little in the base (control) population at least for 10 generations (Takigahira et al. 2014). The resistance against *L. victoriae* KK may

incur some costs and then may be selected against in the base population, or the resistance genes of the BG and KK populations may be linked with some deleterious genes. The AFLP analysis on the original IR, KK and BG populations and the resistant (i.e., selected for increased resistance) and susceptible (control) populations produced in our previous selection study will provide some cues to understand the genetic changes that had occurred in our experiments and further to determine DNA regions associated with the resistance against *L. victoriae* KK.

II-2. Materials and methods

II-2-1. Cross experiments

Laboratory populations of *Drosophila bipectinata* originated from Kota Kinabalu (KK) and Iriomote-jima (IR) were used for cross experiment. F_1 flies were produced by reciprocal crossing of 10 virgin females and 10 males of the IR and KK populations; backcross flies were produced by crossing 10 virgin F_1 females or 10 F_1 males with 10 IR individuals; F_2 individuals were produced by crossing 10 F_1 females and 10 F_1 males (in this cross F_1 individuals from reciprocal crosses were pooled).

To determine the resistance of parental, F_1 and backcross individuals against *L. victoriae* KK, approximately 30 two-day-old larvae and several parasitoid females were placed in a Petri dish containing a small amount of *Drosophila* medium, and the parasitoid females were monitored for oviposition under a stereoscopic microscope. Characteristic oviposition behaviors such as full extension of the ovipositor after contact with the host

and insertions of the ovipositor into the larvae for extended duration (>10 s) were used as indicators of successful oviposition (Vet and Bakker 1985; van Lenteren and Bakker 1978). When oviposition was confirmed, the parasitized fly larvae were transferred into vials containing Drosophila medium. The vials were later checked for the emergence of flies and/or parasitoids. One hundred parasitized larvae were prepared for each cross. Experimental individuals were maintained on Drosophila medium under 15L: 9D (15 h light: 9 h dark) at 23°C. To test deviation from expectations by Menderian inheritance model, chi-square test and likelihood ratio test were performed using R version 2.10.1(R Development Core Team 2009).

II-2-2. Amplified fragment length polymorphism analyses

AFLP analysis was conducted using the original IR, KK and BG populations and the resistant and susceptible populations produced by artificial selection from a base population that was established by mixing the three original populations (Chapter I). At the 10th generation, the rate of successful parasitism by *L. victoriae* KK was less than 10% in selected (resistant) populations and approximately 85% in control (susceptible) populations (Chapter I).

Total genomic DNA was extracted from a total of 98 flies (12 from each of the original populations, 16 from each of the selected populations and 15 from each of the control populations) with the standard phenol-chloroform extraction protocol. AFLP analysis was carried out using AFLP Plant Mapping Kit (P/N 402273, 402005, 4303051, Applied Biosystems, Foster City, USA), according to the manufacturer's recommended protocol. Selective amplification was performed using six primer combinations,

EcoRI+AC / MseI+CTA, EcoRI+TC / MseI+CTT, EcoRI+TA / MseI+CTC, EcoRI+AA / MseI+CAA, EcoRI+AT / MseI+CAG, EcoRI+TT / MseI+CAC. Twenty-four DNA extracts were chosen randomly and subjected to AFLP analysis twice to check the reproducibility, as recommended by Bonin et al. (2004).

Amplified products were analyzed using an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems) with Gene-scan ROX-500 size standard (Applied Biosystems). Raw electropherograms were analysed using PeakScanner version 1.0 (Applied Biosystems) and a binary matrix of AFLP band presence (1) – absence (0) was built using the automated scoring RawGeno package version 2.0-1 (Arrigo et al. 2010) in R with the following parameters: scoring range, 100–500 bp; minimum intensity, 80 rfu; reproducibility limits 85%; minimum bin width, 1 bp; maximum bin width, 2 bp. The percentage of polymorphic loci and the expected heterozygosity (H_j; equivalent to Nei's gene diversity: Nei 1973) were computed for each population with AFLP-SURV version 1.0 (Vekmans 2002). Nei's genetic distances (Nei 1972) and 1000 bootstrapped distance matrices were generated with AFLP-SURV and used to construct neighbor-joining trees and a bootstrap consensus tree with PHYLIP 3.69 (Felsenstein 2010). We also calculated F_{ST} values among populations and population-pairwise F_{ST} with AFLP-SURV to assess the genetic differentiation between populations. Significance of the F_{ST} values was examined from 1000 permutations. Furthermore, we computed the indices of genetic diversities and genetic differentiation using the data set without the outlier locus detected in the following BayeScan analysis (described below).

Detection of outlier loci between the selected and control populations was carried out using BayeScan version 2.0 (Foll and Gaggiotti 2008). BayeScan decomposes F_{ST} into a population-specific component (β) shared by all loci and a locus-specific

component (α) shared by all the populations, and estimates the F_{ST} coefficients using a logistic regression. Departure from neutrality at a given locus is assumed when the locus-specific component is necessary to explain the observed pattern of diversity (i.e., α is significantly different from 0). With a reversible-jump MCMC algorithm, BayeScan estimates the relative posterior probabilities for models with and without selection (α component being either present or absent, respectively), for each locus. The threshold for considering a locus under selection was set according to a Bayes factor (BF) criteria defined by Jeffreys (1961). BF is the ratio of the posterior probability of the selection model to that of the neutral model. In this study, we considered the locus is under selection when the BF value exceeds 32, corresponding to a posterior probability of 0.97, with false discovery rate (FDR) of <0.05. BayeScan was run with 20 pilot runs with length of 5000 iteration each, and an additional burn-in of 50 000 iterations followed by 50 000 iterations with the thinning interval of 10.

II-3. RESULTS

II-3-1. Cross experiments

Figure II-1 shows the proportion of parasitized *D. bipectinata* larvae from which flies, parasitoids and neither of them emerged. In the following analysis, parasitized larvae from which neither of fly nor parasitoid emerged were excluded. In the KK population, the proportion of parasitized larvae from which flies emerged (i.e., fly emergence) was 0.985, whereas 0.075 in the IR population. From parasitized F_1 individuals, no parasitoid

emerged irrespective of the direction of crosses, indicating that the resistant trait is dominant. According to the Mendelian inheritance model with a single locus, the proportion of fly emergence is expected to be 0.795 (=(0.985+0.075)*3/4) in F_2 individuals, and 0.530 in backcross individuals. In the results, the proportion was 0.848 in F_2 individuals, 0.544 in individuals from backcross ((KK♀×IR♂)× IR♂),0.631 in those from backcross ((IR♀×(KK♂× IR♂)), 0.716 in those from backcross ((IR♀×KK♂)× IR♂), and 0.571 in those from backcross ((IR♀×(IR♂× KK♂)). No significant difference was observed between the expectation and the results (χ^2 test, P>0.05), except the result of backcross ((IR♀×KK♂)× IR♂).

The number of host individuals from which neither fly nor wasp emerged was significantly fewer in hybrid (F_1, F_2 and backcross) individuals than the parental KK and IR populations (Likelihood ratio test, -2lnL = 94.00, df = 1, P < 0.001).

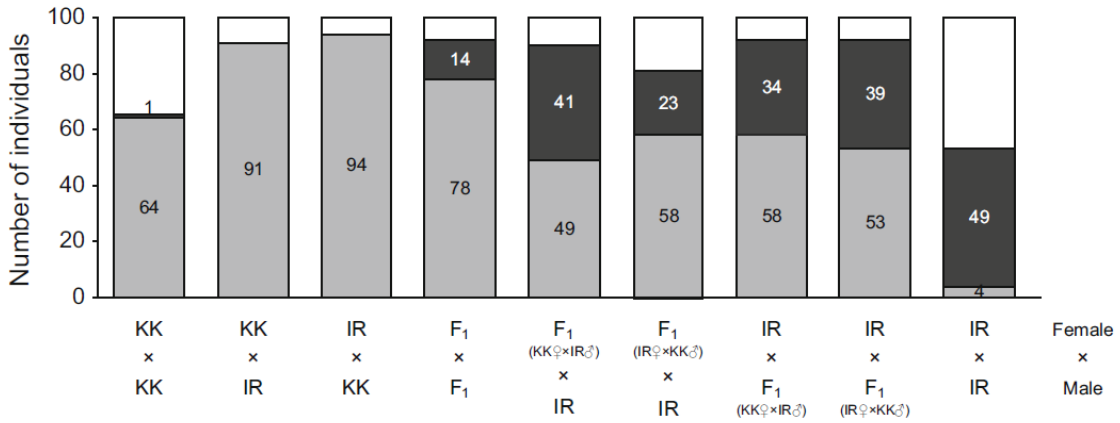


Fig. II-1 The resistance of the the IR and KK populations of D. bipectinata and their F1, F2 and backcross individuals against L. victoriae KK. Numbers of parasitized larvae from which flies (gray), wasps (black) and neither of them emerged (white) were shown.

II-3-2. Amplified fragment length polymorphism analyses

A total of 186 polymorphic AFLP markers were obtained from 93 individuals (BG: 12, KK: 12, IR: 11, S1: 15, S2: 15, C1: 14, C2: 14) using six primer combinations with the error rate per locus of each primer-combination was 0.020 to 0.047. The observed F_{ST} value among populations based on AFLP markers was 0.3548 *(*$P < 0.001$), and the pairwise F_{ST} values ranged from 0.0265 to 0.5606 (TableII-2). Only little difference was observed in the percentage of polymorphic loci and expected heterozygosity ($H_j \pm$ SE) between the selected and control populations (TableII-1). The percentage of polymorphic loci was higher in the selected and control populations compared with the original populations except for the KK population (TableII-1).

According to the pairwise F_{ST} and Nei's genetic distance, the selected and control populations were more closely related to the IR population compared with the BG and KK populations (Fig. II-1). In comparison with the control populations, the selected populations were somewhat closer to the BG and KK populations. In the outlier analysis using BayeScan, only one of all AFLP loci was detected as outlier (fragment length of 276 bp, primer combination: *Eco*RI+TC/*Mse*I+CTT) between the selected and control populations (Fig. II-3). This fragment was found in 26 out of 28 individuals of the control populations, but only in two out of 30 individuals of the selected populations. All of IR individuals possessed this fragment, but only one out of 12 BG individuals and none of 12 KK individuals possessed this fragment. Exclusion of the outlier locus resulted in only small differences in the estimates of the genetic diversity and differentiation indices (Table II-1).

Table II-1. Indices of genetic diversity and population pairwise F_{ST} values estimated from the AFLP data. Upper and lower triangle in pairwise F_{ST} indicates the values include all loci and exclude outlier loci.

Population	N	All Loci				Exclude outlier				Pairwise F_{ST}						
		N_{loci}	$N_{poly.}$ (%)	H_j	S.E. (H_j)	N_{loci}	$N_{poly.}$ (%)	H_j	S.E. (H_j)	BG	KK	IR	S1	S2	C1	C2
BG	12	186	79 (42.5)	0.164	0.0131	185	79 (42.7)	0.164	0.0132		0.489	0.561	0.477	0.450	0.469	0.496
KK	12	186	95 (51.1)	0.198	0.0141	185	95 (51.4)	0.200	0.0141	0.490		0.485	0.392	0.411	0.439	0.439
IR	11	186	80 (43.0)	0.126	0.0113	185	79 (42.7)	0.126	0.0113	0.556	0.478		0.135	0.155	0.034	0.038
S1	15	186	89 (47.8)	0.160	0.0125	185	89 (48.1)	0.161	0.0125	0.478	0.392	0.112		0.031	0.098	0.087
S2	15	186	100 (53.8)	0.172	0.0122	185	99 (53.5)	0.172	0.0123	0.451	0.412	0.136	0.031		0.084	0.078
C1	14	186	92 (49.5)	0.167	0.0113	185	91 (49.2)	0.167	0.0114	0.463	0.432	0.035	0.077	0.067		0.027
C2	14	186	93 (50.0)	0.158	0.0112	185	92 (49.7)	0.156	0.0110	0.497	0.438	0.034	0.080	0.073	0.024	

N_{loci}, number of loci; $N_{poly.}$, number of polymorphic loci; H_j, expected heterozygosity or Nei's genetic diversity.

37

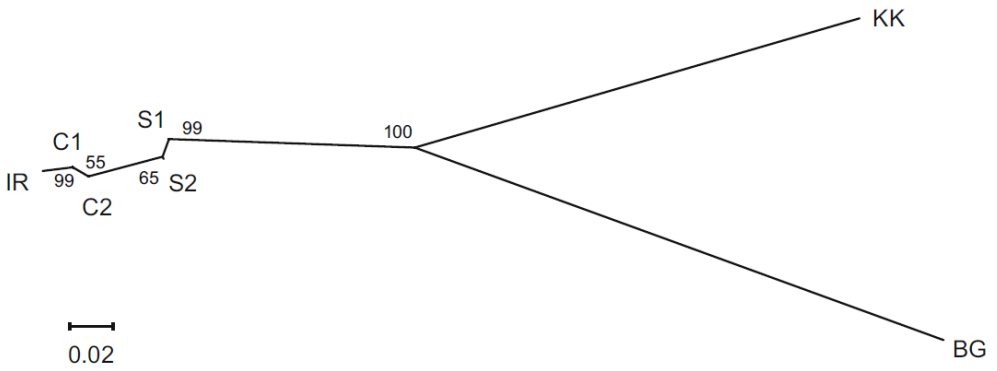


Fig. II-2 Neighbor-joining tree based on Nei's genetic distance among the original populations and the selected and control populations in AFLP analysis. Numbers near nodes are bootstrap support values.

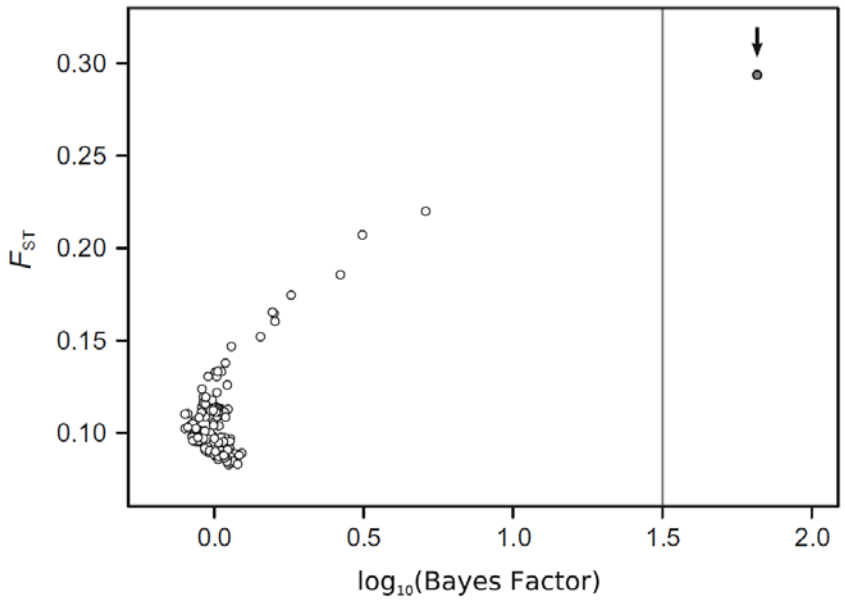


Fig. II-3 Fst value against common logarithm of Bayes Factor obtained by BayeScan. Vertical solid line indicate threshold for loci to be considered under selection (Bayes factor of 32 corresponding to a posterior probability of 0.97). Detected outlier between the selected and control populations is shown with filled circle and indicated by an arrow.

II-4. Discussion

The cross experiments revealed that the difference in resistance against *L. victoriae* KK between the IR and KK populations of *D. bipectinata* was due to alleles on a single locus or few closely linked loci on autosome and the resistance was a dominant trait. The outlier analysis on AFLP markers also detected a single outlier which was almost specific to susceptible flies. However, it is still uncertain whether this outlier is associated with the gene(s) controlling the resistance/susceptibility to *L. victoriae* KK. The molecular analysis of this outlier is a next step for identification of the resistance/susceptibility gene(s) in *D. bipectinata*.

The control of resistance/susceptibility to parasitoids by one or a few loci with large effect has also been suggested for the resistance/susceptibility of *D. melanogaster* and *D. yakuba* against *L. boulardi* (Carton et al. 1992; Dupas et al. 1998, 2003, 2009; Hita et al. 2006; Dubuffet et al. 2007, 2009). In addition, Mortimer et al. (2012) found that a gene in the N-glycosylation pathway regulates the level of encapsulation responses of *D. melanogaster* against *L. clavipes*. However, the resistance gene of *D. bipectinata* against *L. victoriae* KK would differ from those of *D. melanogaster* against *L. boulardi* or *L. clavipes*, because *D. bipectinata* did not show encapsulation response against *L. victoriae* KK (T. Takigahira, personal observation).

In the cross experiments, it also appeared that the number of host individuals from which neither fly nor wasp emerged was fewer in hybrid (F_1, F_2 and backcross) individuals compared to the parental KK and IR populations. This may be attributable to heterosis or hybrid vigor; i.e., hybrid host larvae may be more resistant to stresses due to parasitization and survive better at least to the pupariation, although they may be killed

by parasitoids thereafter. However, hybrid vigor has not so far been reported in the cross experiments on the resistance/susceptibility to parasitoids as far as we know.

The present study also revealed the occurrence of laboratory selection on genes from the IR, KK and BG populations of *D. bipectinata*. In Chapter I, we have reported that two resistant populations were produced from a mixture of the IR, KK and BG populations by artificial selection (Takigahira et al. 2014). In the present AFLP analysis, it appeared that genes from the IR population were more frequently retained in these selected populations and also in the control populations than those from the KK and BG populations, suggesting that at least a number of genes from the IR population are advantageous under laboratory conditions. In addition, the genetic differentiation between the selected (i.e., resistant) and control (i.e., susceptible) lines was low, suggesting that most genes are unrelated and/or unlinked with the resistance against *L. victoriae* KK. These results agree with the result of cross experiments that the resistance is controlled by a single or a few loci. In addition, these results would explain our previous results that the resistance against *L. victoriae* KK was lowered during six generations of free mating (Takigahira et al. 2014); i.e., the resistance gene(s) from the KK and BG populations would have been linked with some genes that were disadvantageous under the laboratory conditions. In the mixed population, the linkage between the resistance gene(s) and low-fitness genes would have been broken during the six generations of free mating, and therefore the resistance would have remained rather stable after the sixth generation (Chapter I).

It is not known what causes genes from the IR population to have advantages over those from the KK and BG populations. Sgrò and Partridge (2000) reported that competitive ability is selected in laboratory culture. However, no significant difference

was observed in the competitive ability between the IR, KK, BG, selected and control populations in our previous study (Chapter I). On the other hand, Houle and Rowe (2003) suggested that flies that began development earlier were advantageous in laboratory culture, because they were reliably able to eclose before transfer to the next bottles. In this respect, the IR population showed faster egg-to-adult development than the KK and BG populations (Chapter I), and therefore it may be advantageous under the laboratory conditions. In fact, the selected and control populations also showed faster development (Chapter I). However, this would not be the only cause of the prevalence of genes from the IR population.

Chapter III

Association between host sex and sexual dimorphism of a larval-pupal endoparasitoid *Leptopilina ryukyuensis*

III – 1. Introduction

Sexual dimorphism, a phenomenon where the two sexes of the same species exhibit phenotypic differentiation beyond the differences in their sexual organs, is commonly observed in a broad range of animal taxa. One of the most frequently studied sexual dimorphisms is sexual size dimorphism (SSD). Males tend to have larger body size than females (male-biased SSD) (Selander 1972; Ralls 1977; Isaac 2005) whereas females tend to be larger than males (female-biased SSD) in poikilothermic vertebrates and invertebrates (Shine 1979; Fairbain 1997; Blanckenhorn et al. 2007; Stillwell et al. 2010). Especially among insects, female-biased SSD is dominant as evidenced from the fact that 72-95% of species within most of major insect taxa exhibit female-biased SSD (Stillwell et al. 2010). Theoretical studies suggest that fecundity selection is one of the important factors that drive the evolution of SSD and this idea is supported by empirical studies (Jones 1982; King 1987; Shine 1988; Van den Assem et al. 1989; Heinz 1991). The proximal factors that generate SSD is the sex-dependent extension of developmental period in order to acquire more resource or sex-dependent variation in growth rate (Slansky 1993). As for the sexual dimorphism in developmental period (sexual development time dimorphism or SDTD) in insects, females often tend to show longer

developmental period than males (female-biased SDTD) resulting in female-biased SSD (Blanckenhorn 2007; Teder 2014).

Among insects, parasitoid wasps are known to show strong female-biased SSD and SDTD (Hurlbutt 1987; MacKauer 1996; Teder and Tammaru 2005). Generally, larvae of parasitoid wasps are completely dependent on a host insect for the nutrition necessary to complete their development, and whether the host insect continues to grow after the infection may be an important factor that determines the host-wasp interaction. In the case of idiobiont parasitoids (idiobionts) that utilize non-growing hosts as their resource (Askew and Shaw 1986; Vinson 1988), host selection by adult female wasps plays an important role to generate SSD: ovipositing female eggs to large hosts and male eggs to small hosts (Schmidt and Smith 1985; Waage and Godfray 1985; Takagi 1986; Strand et al. 1988; Hardy et al. 1992). So far, although SSD and SDTD were extensively studied in idiobionts, little is known about SSD and SDTD in koinobiont parasitoids (koinobionts) that utilize growing host insect as their resource. Koinobionts in general prefer to oviposit on the hosts at an early developmental stage aiming at their immature behavioral and immunological defense against infection (Slansky 1986; Pennacchio and Strand 2006). Because hosts infected by koinobionts often continue to develop and grow (Askew and Shaw 1986; Vinson 1988), the body size of the hosts at the time of infection does not determine the amount of the resource available for the wasps. Additionally, as suggested in many studies, infection by koinobionts changes the developmental period and final body size of the hosts (Slansky 1986; Pennacchio and Strand 2006). For koinobionts, therefore, developmental period and final body size of the hosts at the time when it is consumed by the wasps are expected to be a crucial factor that regulate wasp development (Harvey 2005), suggesting that hosts are also major determinants of SSD and SDTD. If

hosts themselves exhibit sexual developmental differentiation under infection, it might largely influence on the aspect of SSD and SDTD of their koinobionts.

In this study, I examined the effect of host sex on SSD and SDTD of koinobiont parasitoid using *Drosophila albomicans* Duda (Diptera: Drosophilidae) and *Leptopilina ryukyuensis* Novković and Kimura (Hymenoptera: Figitidae) as study materials. *L. ryukyuensis* is one of the most dominant solitary koinobiont endoparasitoids of frugivorous *Drosophila* flies in the Ryukyu archipelago, located in the southern part of Japan (Novković et al. 2012). The wasp infects at the larval stage of drosophilids and the wasp larva continues to develop within the host body until it consumes it completely at the host pupal stage and pupates inside the host puparium. *L. ryukyuensis* exhibits female-biased SSD and SDTD, which is typical in parasitoids. *D. albomicans* is one of the major host species of *L. ryukyuensis* in nature (Novković et al. 2012) and exhibits female-biased SSD (Kitagawa et al. 1982). Here, I examined development of each sex of *D. albomicans* under infection of either female or male of *L. ryukyuensis* and its effect on aspect of SSD and SDTD of *L. ryukyuensis*. In addition, I also examined whether the wasp allocate offspring sex depending on host sex that may maximize its fitness if host sex differently influences on wasp development. Furthermore, host feeding rate were compared to examine whether the behavioral alteration account the host growth under infection.

III – 2. Materials and methods

III-2-1. Insects and rearing conditions

Laboratory stock of *Leptopilina ryukyuensis* and *Drosophila albomicans*, originally collected from *Iriomote-jima* (24.2°N, 123.8°E), were given by Dr. Kimura in Hokkaido University. Both stocks were established from several females and males of each species collected in 2006 and maintained by mass-mating of about 20 females and males for a decade prior to this study. Stocks were reared on Drosophila medium (50 g of cornmeal, molt and sugar, 40 g of dry yeast and 10 g of agar per 1100 ml water) under 15:9 hour light:dark cycle at 23°C. *L. ryukyuensis* was maintained using larvae of *Drosophila simulans* Sturtevant originated from Sapporo (43.1°N, 141.4 E) as host species. All the following experiments were conducted under constant light at 23°C with Drosophila medium described above.

III-2-2. Host and wasp development

About 10 mated females of *D. albomicans* aged one to two weeks after eclosion were transferred with about ten males at the same age into a vial containing the medium and were allowed to oviposit for six hours. After the adult flies were removed, their offspring were reared in the vial until growing up to second-instar and subjected to the following infecton or control treatment. In infection treatment, about 30 second-instar larvae of *D. albomicans* were collected in a Petri dish (35 mm diameter) with a small amount of the fly medium. Then, several mated females of *L. ryukyuensis* aged three to five days after

eclosion were introduced into the dish for infection. Once a wasp started to attack a host larva, other wasps were removed and then the dish was monitored under a stereo microscope, SZ61 (Olympus, Tokyo, Japan), to confirm the infection to the host larva. When the wasp had inserted ovipositor into a larva for more than 10 seconds and pulled it out with screwing, we judged that the larva was infected. For each female wasp, ten infected larvae were collected and were transferred in to a new vial containing the fly medium. Totally twenty-two replicates were set up. To minimize the variation in infection time among the wasps, all the infection treatment was performed for the host larvae aged 42 to 54 hours after the oviposition. In control treatment, ten second-instar host larvae were randomly chosen and transferred into new vials during 42 to 54 hours after oviposition. Six replicates were set up for the control treatment. When host larvae grew up to third-instar and started wandering behavior (Sokolowski et al. 1984), the larvae were sexed under the stereo microscope, and the female and male larvae were transferred into different vials with the fly medium and wet tissue paper. Then I checked if the larvae pupated every six hours, recorded the time of host pupation (host developmental period), and individually transferred each of the pupae into a well of 96-well PCR plate with a piece of wet tissue paper. and checked either emergence of host or wasp or not. I checked the emergence of the host flies or the wasps every six hours. Emerged wasps and their host puparia were stored in 70% ethanol.

III-2-3. Assessment of host and wasp body size

I captured the images of wasp and host puparium samples with a stereo microscope, SZX16 (Olympus) using a CCD camera, DP25 (Olympus). As an index of wasp body

size, thorax length (Appendix. III-1 A) was measured using ImageJ. For host puparium size estimation, the length and width of the puparium (Appendix. III-1 B) were measured. As an index of host puparium size, pupal volume was estimated by approximating to spheroid as $PV = 1/6\,\pi \cdot PL \cdot (PW)^2$, where *PL* is length and *PW* is width of a puparium.

III-2-4. Measurements of host feeding rate

To examine the observed host growth difference was due to the difference in host behavior associated with feeding, feeding rate was measured. The 50 to 80 infected or uninfected second-instar larvae obtained from the infection or control treatment were reared together in a vial. For host larvae aged 78 to 90 hours after the oviposition, the frequency of cephalopharyngeal sclerite retractions of the host larvae (early third- instar) was counted as an indicator of feeding rate (Joshi and Mueller 1988). Each of the infected or uninfected host larva was transferred into a petri dish containing 3% agar coated with a 10% dry yeast suspension (10g yeast in 100 ml water) on its surface and acclimate to test environment for 30 seconds. Then, the number of cephalopharyngeal sclerite retractions for one minute were counted twice with 30 seconds interval under the stereo microscope. After the measurements, the larvae were individually transferred into a well of 24 well plate containing Drosophila medium and reared for 24 hours. Then, the larvae were sexed and transferred into a new vial with Drosophila medium and reared until pupation. Host pupa were individually transferred into a well of PCR plate with a piece of wet tissue paper and reared until eclosion to check successful infection and wasp sex.

III-2-5. Data analyses

The host pupal volume, feeding rate (the mean counts of the cephalopharyngeal sclerite retractions of the two measurements), and wasp thorax length were analyzed using Bayesian liner mixed models. On the other hand, the developmental period of the host (period from host oviposition to host pupation) and the wasp (period from parasitoid oviposition to parasitoid emergence) was analyzed using Bayesian survival (semi-semiparametric proportional hazard) models. In the analyses of host growth and feeding rate, infected and uninfected individuals were analyzed separately. Host sex was included as a fixed variable and experimental replicates were included as a random variable in the models for the infection and the control treatment, whereas wasp sex and its interaction with host sex was included as a fixed variable in the models for infection treatment. In the analyses of wasp phenotypes, host sex, wasp sex, and their interaction were included as fixed variables and experimental replicates as were included as a random variable the in models. The parameter estimation in both Bayesian models are based on sampling from posterior distributions of the parameters using Markov chain Monte Carlo methods. I ran three independent Markov chains in which 2,000 values were sampled with a 1,000 iteration interval, after a burn-in of 5,000 iterations, yielding a posterior sample size of 6000. The convergence of the Markov chains was checked based on Gelman and Rubin convergence diagnostic (Gelman et al. 2003) for each parameter by comparing the variance within each chain and among chains. The median and 95% Bayesian credible interval (95%CI) for each factor were evaluated based on its posterior samples. When 95% CI for a factor did not include zero, the factor is concluded that had statistically

significant impact on response variable. Further, to compare the host development among groups which had different sex (either female or male) and infection status (unparasitized, parasitized by female wasps or parasitized by male wasps), ANOVA for size and log-rank test for developmental period, where group is predictor variable, were conducted. *P*-value was adjusted by Tukey's HSD methods and Holm methods respectively. In addition, within each sex of hosts, the effect of infection on host feeding rate was examined by ANOVA where different infection status is predictor variable. *P*-value was adjusted by Tukey's HSD methods.

To evaluate degree of SSD for host and wasp, one of the size dimorphism indices (SDIs) which was originally proposed by Lovich and Gibbons (1992), was calculated as SDI = [(mean size of the larger sex) / (mean size of the smaller sex)] – 1. The positive values of the SDI indicate that the females were larger than the males while the negative values indicate that the males were larger than females. SDTD was evaluated with the same index using developmental period instead of body size.

The sex ratio in emerged wasp from each host sex were analyzed with Fisher's Exact test (Cox and Snell 1989). The data form 148 wasps emerged in developmental assay and 138 wasps emerged in host feeding assay were merged and were analyzed.

All the statistical analyses in this study were conducted on the software R version 3.3.1 (Team, R. C. 2016). For Bayesian liner mixed model analysis and Bayesian survival analysis, package "MCMCglmm" version 2.25 (Hadfield et al. 2017) and package "spBayesSurv" version 1.1.1 (Zhou et al. 2017) were used in this study.

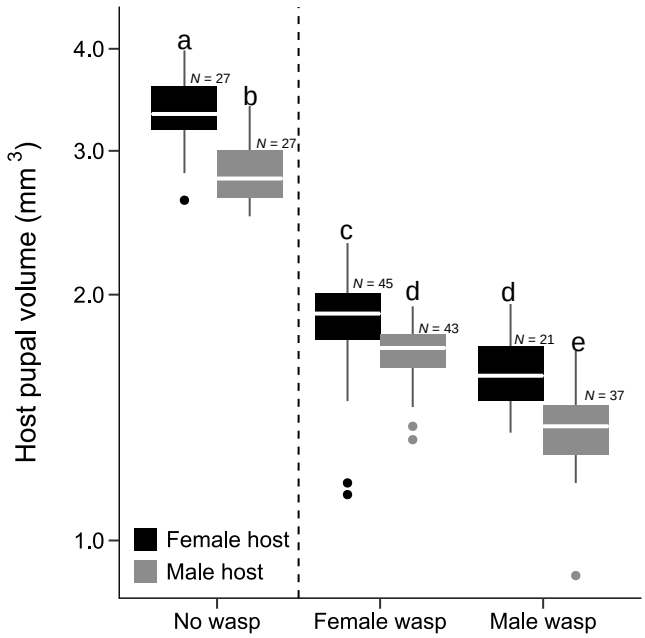
III – 3. RESULTS

III-3-1. Host development

Uninfected hosts showed significant female-biased SSD (SDI = 1.1920) at pupal stage (median = 0.5311; 95%CI [0.3790 to 0.6826]). Infected hosts also showed significant female-biased SSD (median = 0.1702; 95%CI [0.0957 to 0.2453]). Infected wasp sex significantly explained the body size variation within host sex (Median = 0.2583; 95%CI [0.1630 to 0.3520]), and host pupae infected by female wasps were larger than those infected by male wasps (Fig. III-1 A). Interaction between host sex and wasp sex was not significant (median = 0.0514; 95%CI [–0.0682 to 0.1752]), indicating that the degree of host SSD was constant between under female wasp infection (SDI = 1.0967) and under male wasp infection (SDI = 1.1648). All the infected host pupae were significantly smaller than uninfected pupae regardless of the host sex (ANOVA with Tukey HSD; $P < 0.05$) independent of host sex and wasp sex (Fig. III-1 A).

Host sex did not have significant effect on the developmental period regardless of the infection status (uninfected: median = 0.0176; 95%CI [–7.0160 to 7.0587]; infected: median = 0.2740; 95%CI [–0.2600 to 0.8113], Fig. III-1 B). In addition, under infected condition, wasp sex and interaction between host sex and wasp sex did not have significant effect on host developmental period (wasp sex: mean = 0.1166; 95%CI [–0.5292 to 0.7562]; host sex × wasp sex: mean = –0.3674; 95%CI [–1.1779 to 0.4485]). Furthermore, among all the groups, developmental period was not differed (log-rank test: x^2= 10.4, df = 5, p= 0.0641).

(a)



(b)

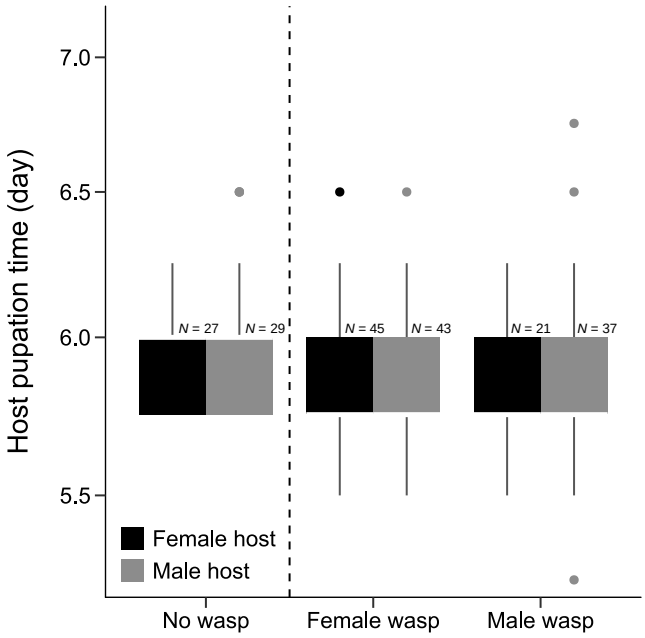


Fig. III-1. Development of hosts that containing no wasp, female or male wasp. A) Host pupal volume. B) Host pupation time. Color of box shows host sex (black: female host, grey: male host). Different letters show significant statistical difference ($P < 0.05$) in multiple comparisons.

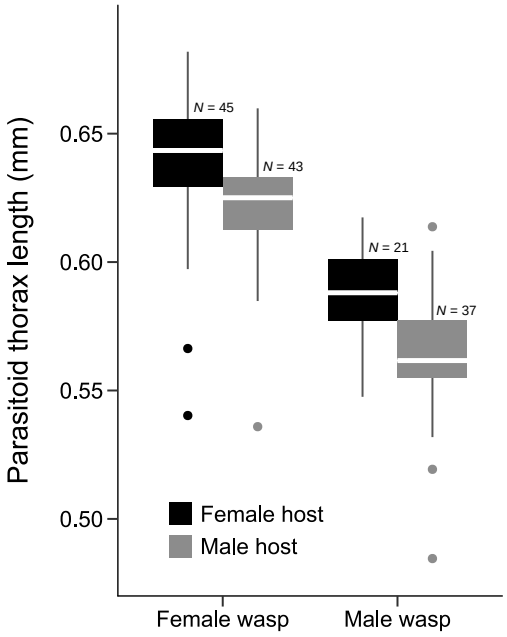
III-3-2. Parasitoid development

From 221 infected hosts, 148 wasps (67.0%) and 23 host flies (10.4%) emerged. On the other hand, neither wasps nor host flies emerged from 50 infected hosts (22.6%); 36 were dead before host pupation and 14 were dead after host pupation.

The wasp body size was significantly affected by its host sex (median = 0.0202; 95%CI [0.0098 to 0.0306]); wasps infected female hosts had larger body size than those infected male hosts (Figure III-2A). Wasp sex further explained the body size variation (median = 0.0508; 95%CI [0.0375 to 0.0644]); female wasps were larger than male wasps. Interaction between host sex and wasp sex was not significant (median = 0.0072; 95%CI [–0.0098 to 0.0242]), indicating that host sex did not influence the degree of wasp SSD. The degree of SSD did not significantly differ between host sex (female SDI = 1.0967, male SDI = 1.1648).

The wasps showed significant female-biased SDTD (median = 1.9433; 95%CI [1.3340 to 2.5597]; Figure III-2B). Host sex and the interaction between host and wasp sexes did not have significant effect on the wasp developmental period (host sex: median = 0.0690; 95%CI [–0.3617 to 0.5036]; host sex × wasp sex: median = –0.0385; 95%CI [–0.7611 to 0.7046]).

(a)



(b)

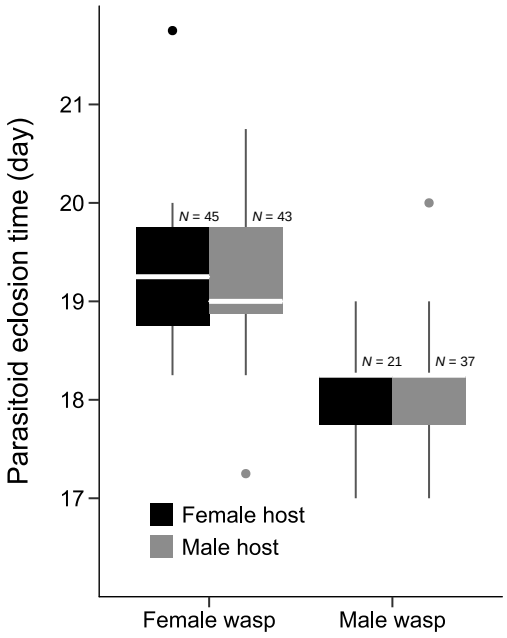


Fig. III-2. Development of parasitoids. A) Parasitoid thorax length. B) Parasitoid developmental period. Color of box shows sex of utilized host (black: female host, grey: male host).

III-3-3. Offspring sex ratio

The percentages of the female wasps emerged from female hosts and male hosts were 69.5% and 62.6%, respectively (Figure III-3). The wasp sex ratios did not differ between female and male hosts (Fisher's exact test: *Odds ratio* = 1.361; *P* = 0.2563).

III-3-4. Host feeding rate

Uninfected hosts did not show the sexual differentiation in feeding rate (median = –2.9074; 95%CI [–0.8392 to 6.6528]), whereas infected hosts showed sexually different feeding rate (median = 6.3754 [–11.8010 to –0.7667); host females showed lower feeding rate than host male under infection. Neither wasp sex nor the interaction between wasp

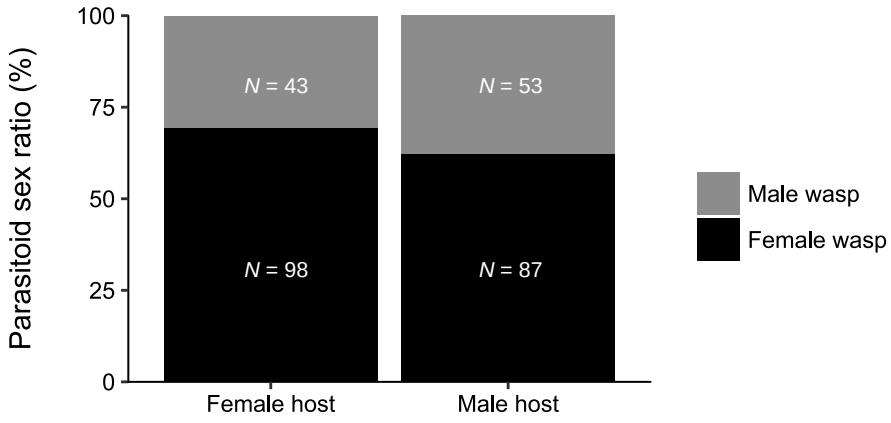


Fig. III-3. Parasitoid sex ratio on each host sex.

and host sexes significantly influence the infected host feeding rate (wasp sex: median = 2.7129 [–3.9252 to 9.2805], host sex × wasp sex: mean = –1.9527 [–8.5217 to 12.6550]). Multiple comparisons revealed that host feeding rate was significantly higher when parasitized by female wasp compared to unparasitized conditions, whereas no significant difference in feeding rate between hosts parasitized by female wasp and hosts parasitized by male, and hosts parasitized by male and unparasitized hosts in both host sex (Figure III-4).

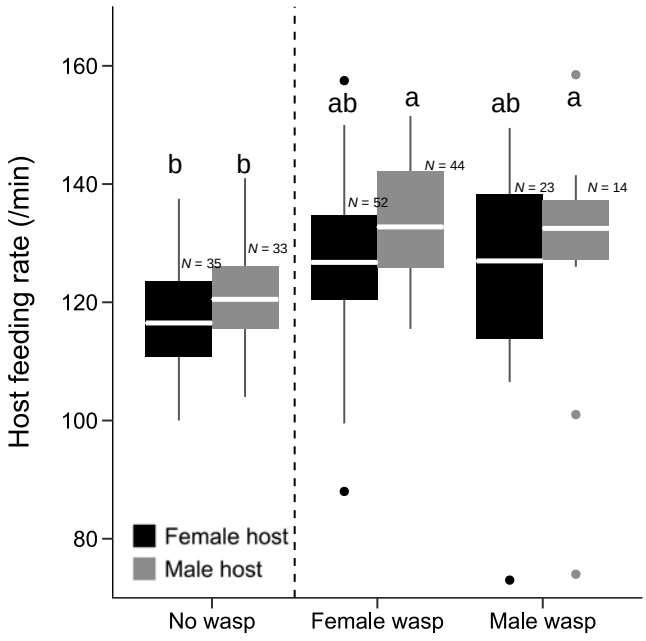


Fig. III-4. Host feeding rate that containing no wasp, female or male wasp. Color of box shows host sex (black: female host, grey: male host). Different letters show significant statistical difference ($P < 0.05$) in multiple comparisons.

III-4. DISCUSSION

In the current study, host flies showed female biased-SSD at pupal stage, but pre-pupal period did not differ between females and males. Infection by the wasps reduced the host pupal size significantly, and the negative effect of the infection differed between infected wasp sex: male wasps showed larger negative effect than female wasps. Wasp infection and the wasp sex did not influence the degree of SSD of the host flies. The current results are similar to the observation in the previous studies on koinobionts that wasp infection did not change the relative growth potential of the hosts (Harvey 2000; Harvey et al. 2010).

Both female and male wasps developed in female hosts attained larger adult body size than those developed in male hosts, reflecting the higher growth potential of the female hosts. In addition, female wasps were larger on average than male wasp regardless of the host sex. As predicted in the previous studies (Slansky 1986; Harvey 2005), wasp size was strongly correlated with infected host pupal size (Appendix. III-2), suggesting that host size is a strong determinant of the wasp size. In insects, generally, female body size is known to more flexibly respond to environmental variation than male body size (Stillwell et al. 2010), and therefore, when a larger amount of the resource is available, larger SSD is expected (Teder and Tammaru 2005). However, although the female host had larger pupal size, host sex did not influence the degree of SSD of the wasps in this study. The current result corresponds to the report by Teder and Tammaru (2005) that parasitoids in general show relatively small plasticity in SSD compared to other types of feeding guilds.

As in many insects, larger parasitoids show higher fitness (Visser et al 1994; Eijs and van Alphen 1999, Ellers et al. 1998), and the fitness gain by the body size increment

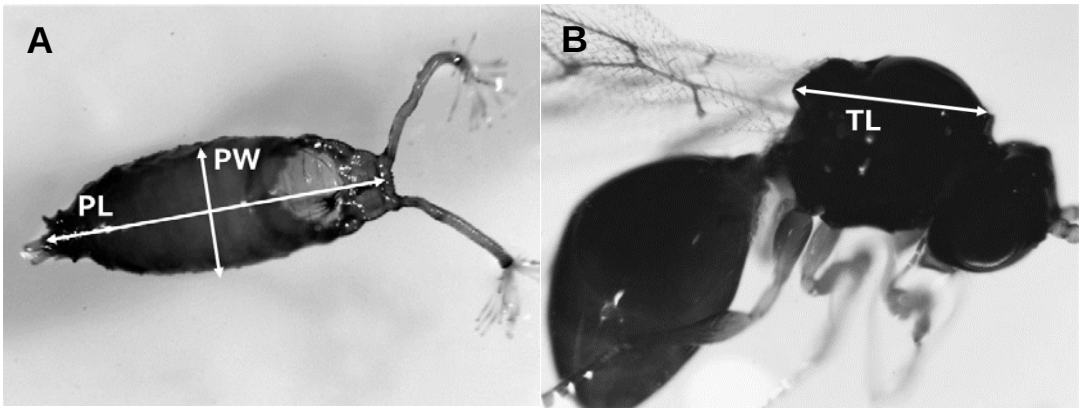
is larger in females (Visser 1994; Gerling et al 1990; Eijs and van Alphen 1999; Ellers et al. 1998; Ellers et al. 2001). Because as parasitoid wasp in general, *L. ryukyuensis* has the potential to allocate offspring sex by haplodiploid sex determination system (Godfray 1994; Quicke 1997), allocating female eggs to female hosts and male eggs to male hosts can be an optimal oviposition strategy for the wasps in this study. However, no evidence of offspring sex allocation depending on host sex was found in this study. Sex allocation depending on host quality evolves widely among idiobionts, where host size at the time of infection determines the resource quality (Schmidt and Smith 1985; Waage and Godfray 1985; Takagi 1986; Strand et al. 1988; Hardy et al. 1992; Mayhew 1998). On the other hand, host growth potential instead of host size at the time of infection can be an important determinant of the body size of koinobionts, but there is no empirical evidence for koinobionts to allocate offspring sex based on the growth potential of the host insects.

The hosts infected by female wasps grew larger than those infected by male wasps resulting in larger resource available for the female wasps. This is a proximal mechanism that generates SSD of the wasps. Similar pattern was also reported in Ichneumonidae koinobionts, *Campoletis sonorensis* and *Diadegma semiclausum* although the sex of the hosts is not taken into consideration (Gunasena et al. 1989; Gols and Harvey 2009). Here I propose two hypothetical mechanisms that explain the observed pattern. Firstly, ovipositing wasps did not discriminate host sex but they might judge host growth potential and allocate female eggs to higher quality hosts as suggested by previous studies. Two koinobioints that infect drosophilids, *Asobara tabida* and *Leptopilina heterotoma*, are shown to discriminate host flies on the basis of infection success rate (van Alphen and Janssen 1982; Strien-van Liempt and Hofker 1985; Van Alphen and Vet 1986). *Ephedrus californicus*, a koinobiont that infects aphids prefer starved hosts regardless of

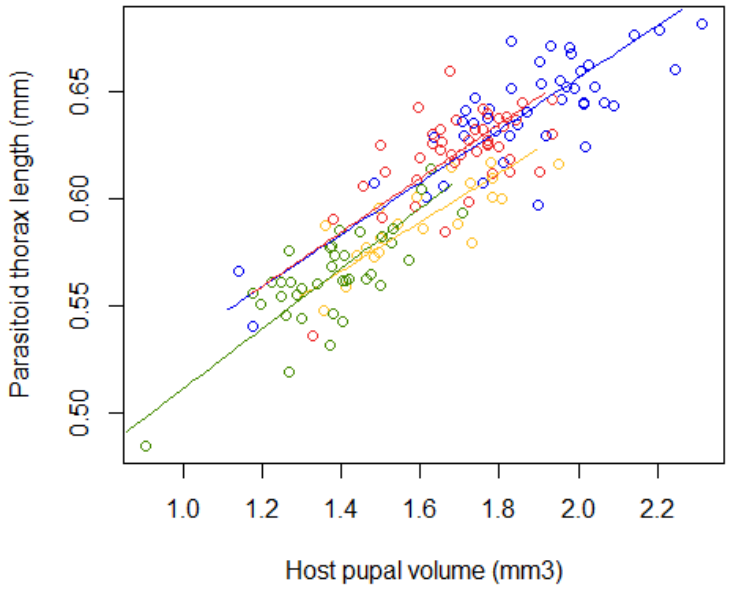
age and size in which they can develop faster than non-starved hosts (Kouamé and Mackauer 1991). Those examples suggest that many koinobioints are able to descrimiate host quality and support the hypothesis described above. Secondly, the wasps may manipulate host growth so that the host body size gets larger when infected with females. Many studies report that koinobiont females inject venoms, virus-like particles (VLPs) and polydanaviruses at oviposition to manipulate host physiology and growth (Vinson and Iwantsch 1980; Coudron 1991; Fathpour and Dahlman 1995; Summers and Dib-Hajj 1995). In the case of ichneumonid endoparasitoids, *Meteorus pulchricornis*, venom and VLPs play a major role to suppress the host immune system and regulate host growth (Suzuki and Tanaka 2006, Suzuki et al. 2008). VLPs are also found in *Leptopilina* species and they suppress host immune system but its effect on host growth was unclear (Rizki andRizki 1990; Morales et al. 2005; Dupas et al. 1996; Labrosse et al. 2003). Future study is necessary to investigate how those two hypothetical mechanisms generate the observed pattern in *L. ryukyuensis*.

The co-occurrence of female-biased SSD and female-biased SDTD was typical in parasitoids (Hurlbutt 1987; MacKauer 1996; Teder and Tammaru 2005). When infecting small hosts, female koinobionts tend to extend host developmental period more to harvest larger amount of resource compared to male koinobionts (Mackauer et al., 1997). In the current study, the female wasps had larger body size than the male wasps, but pre-pupation period of the hosts did not differ when they were infected with female or male wasps, indicating that the female wasps did not extend the developmental period of the hosts. Consequently, SDTD of the wasps came from SDTD after host pupation. Because the hosts infected by the female wasps had larger pupal size, female wasps might have needed longer period of time for consuming and assimilating the host than the male

wasps as in the case of idiobionts (MacKauer 1997; Harvey 2005). Despite the larger host pupal size when infected with female wasps, pre-pupal periods of the hosts infected with female or male wasps did not differ, and developmental period of the wasps after host pupation did not explain this pattern. Therefore, developmental period of the wasps may not be determined by the amount of resource available, but be regulated by known factors. *D. albomicans* showed higher feeding rate under female wasp infection although male wasp infection was not statistically significant, suggesting parasitism effect on host feeding rate. Hosts infected with koinobionts often show altered food intake pattern (Thompson 1982, 1983; Bcntz and Barbosa 1990; Schopf and Steinberger 1996; Shi et al. 2002; Elzinga et al. 2003). However, the difference in the growth rate between the hosts infected with female or male wasps was not due to the difference in their feeding rate, but might be due to the difference in digestion and assimilation processes. Thompson (1983) reported that the infection of *Hyposoter exiguae* increased host assimilation rate and infected host exhibited higher growth rate than uninfected hosts under the same food consumption. Another possibility is that the counts of cephalopharyngeal sclerite retractions in the current study was not a good indicator of the feeding rate or feeding rate might not be constant throughout the developmental period. These limits of the current measurement of the feeding rate might have masked the difference in the feeding rate between the hosts infected with female or male wasps. More appropriate measurement of food intake and behavioral monitoring will reveal the sex-dependent resource accumulation in host of *L. ryukyuensis*.



Appendix. III-1. Measured part of host puparium and parasitoids. A) Host pupal length (PL) and width (PW). B) Parasitoid thorax length.



Appendix. III-2. Association between host pupal volume and parasitoid thorax length. Blue: Female host-Female wasp, red: male host-female wasps, yellow: female host-male wasp, green: male host-male wasp. Host pupal volume and parasitoid thorax length is correlated in all host-parasitoid sex combinations (type II regression: $P < 0.001$).

General Discussion

Interaction between *Drosophila* and parasitoid wasps of genus *Leptopilina* was examined in this thesis. In chapter I and II, fitness cost and genetic control of resistance against parasitoid *Leptopilina victoriae* in *Drosophila bipectinata* were examined. Most of previous studies on resistance in host-parasitoid system involved in *Drosophila melanogaster* and its related species of the member of *Drosophila melanogaster* species subgroup where hemocytes–mediated melanotic encapsulation plays important role in resistance. In *D. bipectinata*, however, melanization was not observed. Such resistance without melanization process was less studied and this is the first study that examined fitness cost of resistance without melanization. The results of current studies suggested that resistance against *L. victoriae* in *D. bipectinata* probably (1) incurs low fitness costs, (2) resistance is specific to certain parasitoid populations or species and (3) singe locus or few linked loci can determine the resistant or susceptible phenotype where resistant phenotype is dominant. These characteristics of resistance is consistent with many previous reports on the resistance in *Drosophila melanogaster* against some parasitoid species (Dupas and Boscaro 1999; Kraaijeveld et al. 2001; Dupas et al. 2009; Mitsui and Kimura 2010; Novković et al. 2012). The results of current studies suggested that evolutionary potential of resistance may be high, i.e., if a host population have variation in resistance, resistance rapidly evolved under strong selection by parasitoid infection, and matched the conditions where coevolutionary arms race between resistance and virulence in host-parasitoids system can occur suggested in the theoretical studies (Doebeli 1997; Sasaki and Godfray 1999). In addition, these characteristics of resistance may also account for the aspect of host-parasitoid interaction in nature; in general,

Drosophila-parasitoid systems are multispecific, i.e., a host species is parasitized by more than one parasitoid species, and a parasitoid species parasitizes more than one host species (Dupas et al. 2009; Mitsui and Kimura 2010; Kimura and Suwito 2012; Novković et al. 2012), and hosts and parasitoids often exhibit resistance and virulence against coexisting multiple species (Novković et al. 2012). If virulence and resistance are specific to a certain host species, such multispecific systems are possible only when virulence and resistance incur low costs; if a resistance to a parasitoid species is costly, it would be difficult to acquire resistance against a number of parasitoids. In general, immune system involved in numerous genes and intensifying immune system incur various fitness costs (Schwenke 2016). However, if host resistance depends on the target recognition of virulent factors by parasitoids, resistance can be changed by simple genetic change and resistance evolution is likely to acquire without costs. Virulence of *Leptopilina* parasitoids is associated with virus like particles (VLPs) which is produced in the venom grand of female wasps and is injected with parasitoid egg in host body. It is known that VLPs bind to host lamellocyte, become internalized and promote lamellocyte lysis resulting in host immune suppression (Marales et al. 2005; Rizki and Rizki, 1984,1990,1992,1994). Therefore, hosts can avoid immune suppression by changing and modifying the target proteins of parasitoid VLPs and such changes may not incur any cost and simply genetic change. Identification of virulence and resistance genes will provide further understanding of parasitoid-host associations in interaction between *D. bipectinata* and *L. victoriae*.

In chapter III, developmental association between *D. albomicans* and *L. ryukyuensis* were examined in aspect of SSD and SDTD. Most of studies on the effect of host characteristics on koinobionts development focused on the difference in host species,

host age (instar) and their size (Ref: Harvey 2005), however, few studies focused on the effect host sex. This is the valuable study that examined interaction between host growth potential associated with their sex (i.e., sexual developmental dimorphisms) and koinobiont development and its sexual dimorphisms. Current study revealed that significant host sex effect on parasitoid development in aspect of size and parasitoids did not allocate host sex depending on the host sex although parasitoid SSD (and also SDTD) ensured may be due to either offspring sex allocation by host condition that independent of host sex or sex dependent growth regulation. These results suggested that evolution of host sexual dimorphisms can strongly influence on development of koinobionts. Because of SSD (and SDTD) is common among insects including *Drosophila* (Blanckenhorn 2007; Stillwell et al. 2010; Teder 2014), it may be one of the most important sources of traits variation for koinobionts in nature. Further analysis in various host–parasitoid interactions provides the evolutionary interaction between host and parasitoid life history traits.

In conclusion, as parasitoids develop as parasites of single host insects which are eventually killed, changes in host and parasitoid population directory influence on antagonist's population and drive the adaptations. Host-parasitoid system is, thus, suitable system for examining various evolution driven by interaction between organisms. Among them, parasitoids wasps focused in this thesis were highly diversified and abundant in terrestrial ecosystems. Therefore, aspect of their interactions and its change will also influence on the evolution of organisms in the community, such as host plants of host insects, or other predators. Accumulating the knowledge about interactions between parasitoids and their host insects will lead to understand the complex ecosystem in nature.

Summary

Chapter I

Theoretical studies predict that strong reciprocal selection in host-parasitoid system resulting in coevolutionary arms race where hosts intensify their immune system to cope with parasitoids (resistance) and parasitoids intensify the ability to parasitization (counter-resistance). One of the important factors that affected such parasitoid-host coevolution is the fitness cost of resistance and counter-resistance. The aim of this chapter was to estimate the fitness cost of resistance evolution in *Drosophila bipectinata* whose resistance differ from *D. meranogaster*, against parasitoid *Leptopilina victoriae*. I established base population for artificial selection by mixing three geographic population of *D. bipectinata*, originated in Bogor (BG), Kota Kinabaru (KK) and Iriomote-jima (IR), BG and KK were resistant whereas IR was susceptible against *L. victoriae* (KK population). After mixing for six generation of free-mating, base population showed low resistance. Then I selected hosts survived under infection in selected treatment whereas randomly picked up hosts in control populations for ten generations. Selected populations showed rapid increase of resistance within four generations, attained high resistance suggesting that the number of genes responsible for the differentiation in resistance might be few. The comparison of life history traits between control and selected populations revealed that only female longevity lowered in selected population whereas male longevity showed opposite trend. Female longevity was also lowered in both BG and KK (resistant) compared to IR (susceptible), and male longevity were higher in KK than BG and IR. In addition, higher resistance in selection populations were maintained after

relaxed selection for 10 generations. These results suggested that resistance might be linked some disadvantageous genes, but its fitness cost may be low. Selection treatment did not affect the resistance against different population of conspecies (*L. victoriae* BG) and different parasitoid species suggesting that resistance in *D. bipectinata* act specific to certain parasitoid populations or species.

Chapter II.

How resistance controlled genetically depends evolutional probability. Rapid increase of resistance in response to selection treatment in chapter I suggested that differentiation in resistance among host population may be determined by few genes. In this chapter, to reveal genetic control of resistance against *L. victoriae* in *D. bipectina* more precisely, I conducted cross experiment and amplified fragment length polymorphisms (AFLP) analysis. In the cross experiment between KK population (resistant) and IR population (susceptible) of *D. bipectinata*, almost all F_1 individuals showed resistant phenotype and phenotype segregation ratio in offspring of backcross between F_1 and IR, and F_2 individuals is almost matched Mendelian inheritance of single locus. Furthermore, in AFLP analyses for three geographic populations, and experimental populations established by artificial selection experiment in chapter I, a single outrider fragment which was almost specific to susceptible flies. This suggested that resistance against *L. victoriae* in *D. bipectinata* is dominant phenotype and differentiation between populations may be determined by a single locus or few closely linked loci on autosome. In addition, in cross experiment, the rate of dead individuals (i.e., parasitized individuals

from which neither fly nor wasp emerged) was fewer than hybrid individuals than parental populations, suggesting that effect of heterosis on the stress tolerance to parsitization of host independent of its fate. AFLP analysis further revealed that genes from IR population, which susceptible to *L. victoriae*, were much retained among both control and selected populations, and genetic differentiation between control and selected population were low. These results suggested that at least number of genes from the IR genes are advantageous under laboratory conditions and most of genes were unrelated and/or unlinked with the resistance. the results of AFLP analysis suggested that resistance gene(s) from the BG and KK populations would have been linked with some disadvantageous but the linkage between the resistance genes and low-fitness genes would have been broken during free-mating for making base population.

Chapter III.

Parasitoid wasps are known to show strong sexual dimorphisms in body size (SSD) and developmental period (SDTD) where female is larger and have longer development time than male. This is considered because female enjoys more fitness by increase in size than male and needs to longer development time to acquire more nutritional resource. In koinobionts, which allow their host to grow in size after parasitization, developmental period and final body size of the hosts at the time when it is consumed by the wasps are predicted to be a crucial factor that regulate wasp development, suggesting that hosts are also major determinants of SSD and SDTD. However, how host growth potential influences the aspect of koinobiont SSD and SDTD is less understood. In this chapter, I

examined host sex effect on the koinobiont sexual dimorphisms using *Drosophila albomicans* which represent SSD and their larval-pupal parasitoid *Leptopilina ryukyuensis*. First, I compared host development under unparasitized and parasitized condition. This revealed that parasitized *D. albomicans* also shows SSD where female is larger than male suggesting the higher growth potential of the female hosts. In addition, I found that significant parasitoid sex effects on the host size, i.e., hosts of female wasp tended to be large than hosts parasitized by female. I propose two hypothetical mechanisms that explain the observed pattern, 1) wasp discriminates host growth potential independent of host sex or 2) wasp differently regulates host growth depending on offspring sex. Both female and male wasps developed in female hosts attained larger adult body size than those developed in male hosts, reflecting the higher growth potential of the female hosts. In addition, female wasps were larger on average than male wasp regardless of the host sex. These patterns can be accounted by strongly correlation with host pupal size. On the other hand, host sex did not influence on the degree of SSD and SDTD. There was no evidence of offspring sex allocation depending on host sex was found in this study. The female parasitoids showed longer development time than male, but this is not due to extension of host developmental period. Infection of parasitoid influence on the host feeding rate but it did not account the sex-dependent resource accumulation in host of *L. ryukyuensis*.

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