The leafminer Liriomyza trifolii (Diptera: Agromyzidae) encapsulates its koinobiont parasitoid Halticoptera circulus (Hymenoptera: Pteromalidae): implications for biological control

The leafminer *Liriomyza trifolii* (Diptera: Agromyzidae) encapsulates its koinobiont parasitoid *Halticoptera circulus* (Hymenoptera: Pteromalidae): implications for biological control

Taichi Kemmochi, Satsuki Fujimori and Tsutomu Saito*

Faculty of Agriculture, Shizuoka University, Ohya, Suruga, Shizuoka 422-8529, Japan

Short title: Encapsulation of parasitoids in leafminers

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*Author for correspondence

Phone: +81 54 238 4790

Fax: +81 54 238 4790

E-mail: atsaito@ipc.shizuoka.ac.jp

Abstract

The koinobiont parasitoid *Halticoptera circulus* (Walker) is a potential biological control agent of leafminers, but it has only rarely been collected from the invasive leafminer, *Liriomyza trifolii* (Burgess), in Japan. To understand why this is the case, parasitism and development of *H. circulus* in *L. trifolii* was compared with parasitism and development in two indigenous leafminer species, *Liriomyza chinensos* Kato and *Chromatomyia horticola* (Goureau). There was no significant difference in parasitism rates by *H. circulus* in the three leafminer species and the eggs and larvae successfully developed in *L. chinensis* and *C. horticola*. However, *H. circulus* failed to develop in *L. trifolii*, where developmental stages were encapsulated by host haemocytes. This parasitoid may be a good agent to control indigenous leafminers such as *L. chinensis* and *C. horticola* but is unlikely to be useful for the biological control of the invasive *L. trifolii* in Japan.

Keywords: invasive leafminer, *Liriomyza chinensis*, *Chromatomyia horticola*, IPM, immunity

Introduction

The dipteran agromyzid leafminers, *Liriomyza trifolii* (Burgess), *Liriomyza chinensis* (Kato) and

Chromatomyia horticola (Goureau) are important pests of vegetables and ornamental crops (Spencer, 1990). In Japan, *L. chinensis* and *C. horticola* are indigenous species while *L. trifolii* is an invasive species that was first reported in Japan in 1990 (Saito, 1993; Sasakawa, 1993). The pest status of these leafminers is compounded by their lack of susceptibility to insecticides (Parrella & Keil, 1984; Saito *et al*., 1992, 1996, 2008a; Saito, 2004; Tokumaru & Okadome, 2004). A koinobiont parasitoid of leafminers, *Halticoptera circulus* (Walker) (Hymenoptera: Pteromalidae), is also resistant to the broad-spectrum insecticides, malathion (organophosphate) and tralomethrin (pyrethroid), and is, therefore, considered to have great potential as a biological control agent of leafminers within Integrated Pest Management (IPM) systems that include the use of insecticides (Saito *et al*., 2008a; Matsuda & Saito, 2014). However, *H. circulus* is rarely reported from *L. trifolii* in Japan (Saito *et al*., 1996, 2008b; Arakaki & Kinjo, 1998; Amano *et al*., 2008) while it is relatively abundant in *L. chinensis* and *C. horticola* populations (Takada & Kamijo, 1979; Tokumaru, 2006; Saito *et al*., 2008a). To develop successful biological control of leafminers using *H. circulus*, we need to understand the reasons why so few have been reported from *L. trifolii*. The purpose of this study was to assess parasitism and development of *H. circulus* in *L. trifolii* compared to the indigenous leafminer hosts, *L. chinensis* and *C. horticola*.

Materials and methods

Culture of leafminers and the parasitoid H. circulus

The leafminers, *L. trifolii*, *L. chinensis* and *C. horticola*, were collected from tomato, garden pea and leek in Shizuoka Prefecture, Japan, in 1991, 2012 and 2005, respectively. Both *L. trifolii* and *C. horticola* were maintained on potted kidney bean plants at 23 ± 1 °C in a 16L: 8D photoperiod; *L. chinensis*, a specialist on *Allium* spp., was maintained on potted leek plants (about 20 cm height) under the same abiotic conditions. These potted host plants were used throughout the study. The parasitoid *H. circulus* was collected from *L. chinensis* infesting leek plants in Shizuoka and Okinawa Prefecture, Japan, in 2010 and 2013, respectively; they were named Shizuoka strain and Okinawa strain. Both strains were maintained on *C. horticola* larvae on potted kidney bean plants at 23 ± 1 °C in a 16L: 8D photoperiod.

Parasitism and host feeding by H. circulus *on each potential leafminer host*

The Shizuoka strain of *H. circulus* was used in this study. Its parasitism and host feeding were evaluated in two trials each of which had different densities of host larvae. To prepare leafminer larvae as hosts for *H. circulus*, approximately 200 adults of each leafminer species were released into a large cage (40 cm width, 40 cm depth, 40 cm height, with gauze sides) containing five or ten potted host plants for Trial 1 and Trial 2, respectively, for 6 h at 23 ± 1 °C and in light during which time oviposition proceeded. The plants were then transferred to another large cage ensuring that all adult leafminers were removed, and maintained at 23 ± 1 °C in a 16L: 8D photoperiod for seven days during which time leafminer eggs hatched and larvae developed. Then numbers of host larvae were adjusted by removing excess larvae using minute pins; in Trial 1 and 2 there were approximately 60 and 30 third-instar larvae per plant, respectively. The potted host plants were then placed individually into smaller cages (22 cm width, 18 cm depth, 35 cm height, with gauze sides); five adult female and five adult male *H. circulus* (2-day-old) were introduced into each small cage and maintained at 23 ± 1 °C in a 16L: 8D photoperiod for 24 h. All leafminer larvae were then dissected in saline solution (0.8% NaCl) with minute pins under a binocular microscope (MZ16, Leica Microsystems, Wetzlar, Germany) and the presence of parasitoid eggs was determined. Simultaneously, the number of host larvae that had been killed by host feeding was determined; these larvae were recognizable as they had turned brown in colour and there was no evidence of parasitoid eggs or larvae. Percentage data for parasitism and host feeding in each cage were logit transformed and submitted to analysis of variance (ANOVA) followed by Tukey's honesty significant difference (HSD) tests in the software package IBM SPSS Statistics (SPSS, 2009).

Development of H. circulus *in each potential leafminer host at different temperatures*

The Shizuoka strain of *H. circulus* was used in this study. Five potted host plants infested with eggs of each leafminer species were prepared as described in the previous section. The cages were maintained at 23 ± 1°C in a 16L:8D photoperiod for four days during which time leafminer eggs hatched and larvae developed; each plant bore 50 to 80 first instar larvae by the end of this time period. A mixed sex population of approximately 100 adult *H. circulus* (2-day-old) was introduced into each cage and maintained at 23 ± 1°C in light for 6 h during which time oviposition proceeded.

For each cage the leaves were then cut from all plants and divided into three groups. Each group was wrapped in a paper towel, placed in a container (16 cm width, 21 cm depth, 6 cm height) covered with a gauze lid, and maintained at either 15 ± 1, 20 ± 1, or 25 ± 1°C in a 16L:8D photoperiod. Thereafter, the number of emerged female and male parasitoids were counted and removed daily between 15.00 and 16.00 hours. Developmental time (eggs to adults) of the parasitoid were compared statistically using the Student's *t*-test in the software package SPSS (SPSS, 2009).

Encapsulation of H. circulus *in each potential leafminer host*

Both the Shizuoka and Okinawa strains were used in this study. Large cages, each containing five potted host plants infested with third instar larvae of each leafminer species were prepared as described previously. Three large cages were prepared for each leafminer species. A mixed sex population of approximately 50 adult *H. circulus* (2-day-old) was introduced into each cage and maintained at 23 ± 1°C in light for 6 h during which time oviposition proceeded. The plants were then transferred to another large cage ensuring that all adult parasitoids were removed, and maintained at 20 ± 1°C in a 16L: 8D photoperiod. For the Shizuoka strain, after 1, 3, 5, and 7 days, 50 to 60 larval and/or pupal leafminers were sampled from each cage and dissected in 5% saline solution on a glass slide under a microscope (Axio Imager 2, Carl Zeiss Microscopy, Jena, Germany). For the Okinawa strain, dissection was done after 5 days using the same method as described above. The percentage of parasitoid eggs and/or larvae that were encapsulated within parasitized leafminers was determined as an indication of the host's immune response; the data from the three cages for each leafminer species were pooled.

Results

Parasitism and host feeding by H. circulus *on each potential leafminer host*

In Trial 1, parasitism rates by *H. circulus* were 21.6% for *L. trifolii*, 22.3% for *L. chinensis* and 18.6% for *C. horticola* and there was no significant difference in parasitism rates between the species ($F_{2,12}$ = 0.262, P = 0.773; Table 1). In Trial 2, which had about half the initial larval density of Trial 1, parasitism rates were 46.7% for *L. trifolii*, 39.7% for *L. chinensis* and 46.3% for *C. horticola* and there was also no significant difference in parasitism rates between the species ($F_{2,27}$ = 1.180, P = 0.323; Table 1). In both trials, host feeding of the parasitoid was observed and ranged from 14.6–32.8% and 8.9–23.0% in Trial 1 and 2, respectively. The mean percentages of host larvae fed upon by adult parasitoids were significantly different amongst the species in both trials (Trial 1: $F_{2,12}$ = 30.012, P < 0,001, Trial 2: $F_{2,27}$ = 6.007, P = 0.007), but subsequent analysis using the Tukey's HSD test indicated that the significant effects were different in each trial (Table 1).

Development of H. circulus *in each potential leafminer host at different temperatures*

There was no development of adult parasitoids from eggs presumed to have been laid in the leafminer *L. trifolii* at any of the temperatures evaluated (Table 2). In contrast parasitoid eggs developed through to adults in larvae of the leafminers *L. chinensis* and *C. horticola* at all temperatures evaluated; development was significantly faster for both male and female parasitoids in *C. horticola* than in *L. chinensis* at all temperatures ($P < 0.01$, Student's *t*-test; Table 2).

Encapsulation of H. circulus *in each potential leafminer host*

Parasitoid eggs (Shizuoka strain) laid in larvae of the leafminers *L. chinensis* and *C. horticola* successfully developed into larvae within 7 days of oviposition and no encapsulated individuals were observed (Table 3). The parasitoid eggs laid in larvae of *L. trifolii* became encapsulated, either as eggs or as larvae, by host haemocytes (Fig. 1A and B). The first evidence of encapsulation was observed 3 days after oviposition when encapsulation rates of eggs and larvae were 1.1% and 66.7%, respectively (Table 3). After 5 days encapsulation rates of eggs and larvae were 10.2% and 91.4%, respectively, and after 7 days encapsulation rates of eggs and larvae were 5.1 % and 100%, respectively (Table 3). In contrast, parasitoid eggs laid in larvae of *L. chinensis* and *C. horticola* successfully developed into larvae within 7 days of oviposition and no encapsulated individuals were observed (Fig. 1C and D, Table 3).

Encapsulation of the Okinawa strain of *H. circulus* by the leafminers was also examined; the eggs and larvae were encapsulated in larvae of *L. trifolii* 5 days after oviposition, while no encapsulated individuals were observed in larvae of *L. chinensis* and *C. horticola* (Table 4).

Discussion

To understand the reason why there was no emergence of the parasitoid from *L. trifolii*, even though the parasitoid laid eggs in this leafminer species, larvae exposed to the parasitoid were dissected and development of the parasitoid was observed. This showed categorically that the parasitoid eggs laid in larvae of *L. trifolii* became encapsulated, either as eggs or as larvae, by host haemocytes (Fig. 1A and B, Table 3). Encapsulation by *L. trifolii* was found for both the *H. circulus* strains evaluated (from Shizuoka and Okinawa Prefecture) (Table 3 and 4), which had been collected approximately 1300 km from each other. This suggests that encapsulation of *H. circulus* by *L. trifolii* may be a common phenomenon in Japan.

In insects, the innate immune system involves both humoral and cellular responses to foreign bodies, and plays an important role in defence against parasitoids (Strand & Pech, 1995). The most well characterized immune response to the presence of parasitoid eggs or larvae is encapsulation by host haemocytes, and results in death of the parasitoid (Meloche & Guppy, 1990; Lavine & Strand, 2002). The present study showed that eggs and larvae of *H. circulus* were encapsulated in *L. trifolii*, even though the parasitoid accepted *L. trifolii* as a potential host and laid eggs within it. Furthermore, encapsulation of *H. circulus* has been found in another invasive leafminer, *Liriomyza sativae* Blanchard (Diptera: Agromyzidae) (unpublished data), that was first reported in 1999 in Japan (Iwasaki *et al*., 2000). The parasitoid has also only rarely been collected from *L. sativae* in the field in Japan (Saito *et al*., 2008b). In contrast, our study also showed that *H. circulus* successfully developed without any encapsulation when laid in the indigenous leafminers, *L. chinensis* and *C. horticola*. Indeed, the parasitoid is also relatively abundant in both leafminer species in the field (Saito *et al*., 2008a; Tokumaru, 2006). These observations suggest that *H. circulus* is killed by encapsulation as part of the host's immune response, but only in invasive species such as *L. trifolii* and *L. sativae* and not in indigenous species.

Host resistance and parasitoid virulence are coevolved in host-parasitoid interactions (Kraaijeveld *et al*., 1998). In the coevolutionary arms race between parasitoid and host the parasitoid is generally the winner, as a result of low levels of encapsulation (Carton & Nappi, 1991). In Japan, *H. circulus* has a short coevolutionary history with the invasive leafminers, *L. trifolii* and *L. sativae*, thereby immunosuppressive strategies may be underdeveloped against the resistance mechanisms of these leafminers. This hypothesis is supported by the fact that *H. circulus* is abundant in populations of *L. trifolii* and *L. sativae* in Florida (Schuster *et al*., 1991), where *L. trifolii* is indigenous (Minkenberg, 1988). However, when we consider populations of *H. circulus* in *L. trifolii* in other regions of its invasive range, our hypothesis does not hold. For example, *H. circulus* is abundant in *L. trifolii* populations in California (Trumble, 1985), Texas (Hernández *et al*., 2011) and Hawaii (Johnson, 1987), following invasion by *L. trifolii* in the mid-1970s (Minkenberg, 1988). Thus, there is geographic variation in virulence of *H. circulus* to *L. trifolii* that cannot always be explained by the length of their coevolutionary history.

It is known that other factors can be involved in variation in virulence of parasitoids. For example, Kraaijeveld & Godfray (1997) reported that there was a trade-off between parasitoid resistance (encapsulation) and larval competitive ability in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae); populations that were highly resistant to the parasitoid had lower larval survival under conditions of intraspecific competition. Such a trade-off may exist in *L. trifolii*, although the phenomenon has not previously been reported for leafminers and their parasitoids. Kapranas & Tena (2015) also reported that older or larger nymphs of soft scale insects achieved higher encapsulation rates of their parasitoids. However, it is unlikely that the latter factor is responsible for the varied virulence of *H. circulus* against *L. trifolii*, because no emergence of the parasitoid was observed in the leafminer species, even though small (first instar) larvae were used as hosts (Table 2). Further immunological studies are needed to fully elucidate geographic variation in virulence of *H. circulus* to *L. trifolii*.

An additional two results were apparent from this study. Firstly, parasitoids developed significantly faster (both males and females) in *C. horticola* than in *L. chinensis* at all temperatures, which suggests that developmental time of this koinobiont endoparasitoid also depends on the larval and pupal developmentl periods of the host; at 25°C, *C. horticola* has a shorter developmental period (11.2 days) than *L. chinensis* (19.2 days) which supports this hypothesis (Mizukoshi & Togawa, 1999; Tran & Takagi, 2005). Secondly, there were also significant differences amongst the leafminer species in the percentages of larvae that were fed on by the parasitoid (Table 1). These results are difficult to interpret because the significant effects were inconsistent between the trials; as prey density was the only factor that was different it is possible that this influenced feeding preference but this would require further research to confirm.

In conclusion, the present study is, to the best of our knowledge, the first record of encapsulation of *H. circulus* eggs and larvae by *L. trifolii*. This suggests that the Japanese strain of *H. circulus* is unlikely to be useful for biological control of *L. trifolii* in Japan, even though it does kill this leafminer by host feeding. Other strains of the parasitoid may be effective control agents of *L. trifolii* in other countries such as USA and it may be possible to select strains with greater efficacy against *L. trifolii* in Japan. In Japan, the parasitoid could also be useful for biological control of the indigenous leafminer species *L. chinensis* and *C. horticola*.

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Figure Legend

Figure 1. Eggs and larvae of *H. circulus* (Shizuoka strain) in leafminers. An encapsulated egg (A) and larva (B) in *L. trifolii.* A healthy egg (C) and larva (D) in *C. horticola*.

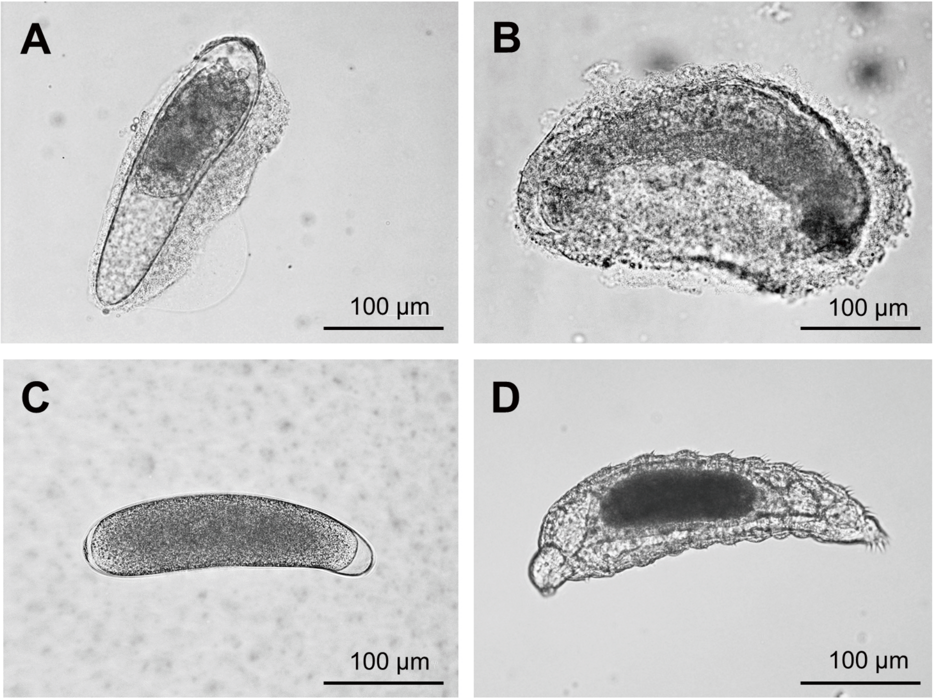
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Table 1. Parasitism and host feeding rates (%, mean ± SE) of *H. circulus* (Shizuoka strain) in each potential leafminer host species.

Host leafminer	Trial 1				Trial 2			
	No. of replicates	Total No. of larvae	Parasitism	Host feeding	No. of replicates	Total No. of larvae	Parasitism	Host feeding
L. trifolii	5	295	21.6 ± 6.4	15.8 ± 1.5 a	10	300	46.7 ± 4.3	23.0 ± 5.5 a
L. chinensis	5	315	22.3 ± 2.8	14.6 ± 1.7 a	10	300	39.7 ± 3.6	8.9 ± 1.6 b
C. horticola	5	298	18.6 ± 3.9	32.8 ± 1.6 b	10	300	46.3 ± 2.8	14.0 ± 2.2 ab

There was no significant difference in the mean percentage of parasitism amongst host leafminer species in Trial 1 ($F_{2,12}$ = 0.262, P = 0.773) or Trial 2 ($F_{2,27}$ = 1.180, P = 0.323). Within the host feeding column, means followed by the different letters are significantly different to each other (P < 0.05).

Table 2. Developmental time (days from egg to adult, mean ± SE) of *H. circulus* (Shizuoka strain) in each potential leafminer host species.

Host leafminer	Sex of *H. circulus*	Temperature (°C)		
		15	20	25
L. trifolii	Female	Not emerged	Not emerged	Not emerged
	Male	Not emerged	Not emerged	Not emerged
L. chinensis	Female	57.2 ± 0.4 **	34.0 ± 0.5 **	23.9 ± 0.3 **
		(*n* = 58)	(*n* = 21)	(*n* = 31)
	Male	55.1 ± 0.3 **	32.6 ± 0.4 **	22.4 ± 0.3 **
		(*n* = 45)	(*n* = 26)	(*n* = 37)
C. horticola	Female	52.0 ± 0.3 **	28.3 ± 0.2 **	20.3 ± 0.2 **
		(*n* = 51)	(*n* = 83)	(*n* = 31)
	Male	50.8 ± 0.4 **	27.4 ± 0.2 **	19.7 ± 0.3 **
		(*n* = 32)	(*n* = 72)	(*n* = 32)

**At each temperature, there was significant difference in the developmental time of male and female *H. circulus* in *L. chinensis* and *C. horticola* ($P < 0.01$). *n* = a total number of parasitoids emerged.

Table 3. Encapsulation rates (%) of eggs and larvae of *H. circulus* (Shizuoka strain) in each potential leafminer host.

Host leafminer	Day 1	Day 3		Day 5		Day 7	
	Eggs	Eggs	Larvae	Eggs	Larvae	Eggs	Larvae
L. trifolii	0	1.1	66.7	10.2	91.4	5.1	100
	(*n* = 90)	(*n* = 95)	(*n* = 3)	(*n* = 49)	(*n* = 58)	(*n* = 39)	(*n* = 26)
L. chinensis	0	0	0	0	0	No eggs	0
	(*n* = 55)	(*n* = 33)	(*n* = 3)	(*n* = 6)	(*n* = 41)		(*n* = 58)
C. horticola	0	0	0	0	0	No eggs	0
	(*n* = 35)	(*n* = 48)	(*n* = 13)	(*n* = 3)	(*n* = 47)		(*n* = 59)

n =number of eggs or larvae observed.

Table 4. Encapsulation rates (%) of eggs and larvae of *H. circulus* (Okinawa strain) in each potential leafminer host at day 5 after oviposition.

Host leafminer	Eggs	Larvae
L. trifolii	57.6	59.5
	(*n* = 99)	(*n* = 80)
L. chinensis	0	0
	(*n* = 3)	(*n* = 85)
C. horticola	0	0
	(*n* = 2)	(*n* = 102)

n = number of eggs or larvae observed.