Diversity and host associations of parasitoids attacking mycophagous drosophilids (Diptera: Drosophilidae) in northern and central Japan

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Abstract

The diversity and host associations of parasitoids attacking mycophagous drosophilids were studied in Tokyo (the warm-temperate region) and Sapporo (the cool-temperate region) in Japan. Field collections were carried out using traps baited with mushrooms in May, June, September and October, 2009 in Tokyo and in July and August, 2010 in Sapporo. The major drosophilid species that emerged from mushroom baits was *Drosophila bizonata* in Tokyo and *D. orientacea* in Sapporo. In total, 13 parasitoid species emerged from drosophilids occurring in mushroom baits, and 11 of them were larval parasitoids belonging to Braconidae and Figitidae. Among the 11 larval parasitoids, 10 were collected in Tokyo, while only two were collected in Sapporo. It is not known why their diversity differed so much between these two regions. Four of the 11 larval parasitoids have also been recorded from drosophilid larvae occurring in fruits (banana). The use of these two habitats (mushrooms and fruits) by these four species seems to reflect the occurrence (i.e. resource use) of their suitable hosts. On the other hand, most larval parasitoids from Tokyo attacked *D. bizonata*, and two larval parasitoids from Sapporo attacked *D. orientacea*, suggesting that the abundance of potential hosts is one of the important factors affecting their host use.

Key words: Braconidae, coevolution, Figitidae, Hymenoptera**.**

INTRODUCTION

Host range of parasitoids is affected by various factors, such as host habitats, host taxonomy and/or competition with other parasitoid species (Askew & Shaw 1986; Askew 1994; Godfray 1994; Hawkins 1994). For example, parasitoids are expected to search for potential hosts in habitats where the availability of suitable hosts is high (Gauld 1988; Whitman & Eller 1990; Hawkins 1994), and therefore insects that occur in other habitats may be seldom attacked by these parasitoids even if they are physiologically suitable as host. In addition, closely related species may be attacked by common parasitoid species, since they would share physiological or morphological characteristics that may determine their suitability as host (Vinson & Iwantsch 1980; Askew 1994; Godfray 1994). The abundance of potential hosts also affects the parasitoid host selection (Hawkins 1994; Sheehan 1994; Sasaki & Godfray 1999; Lapchin 2002); it is beneficial for parasitoids to evolve virulence against frequently encountered (i.e. abundant) potential hosts, whereas it may costly for parasitoids to pursue less abundant potential hosts if they evolved resistance.

We present here researches on the diversity and host associations of parasitoids attacking mycophagous drosophilids in central and northern Japan to determine factors influencing their host use. *Drosophila* species and their parasitoids have been used as a model system for the study of coevolutionary interactions (Fellowes & Godfray 2000; Kraaijeveld & Godfray 2001, 2009; Dupas *et al*. 2009; Prévost *et al*. 2009), but how the host range of *Drosophila* parasitoids is determined is still elusive,

since not many studies on their field ecology have been conducted except for Janssen *et al*. (1988), Driessen *et al*. (1990), Fleury *et al*. (2004, 2009), Yorozuya (2006) and Mitsui and Kimura (2010).

MATERIAL AND METHODS

Field survey

Collections of parasitoids were carried out in Tokyo (35.6 °N, 139.4 °E) and Sapporo (43.1 °N, 141.3 °E) located in the warm- and cool-temperate regions, respectively. Sapporo is located about 60 km north of Tomakomai where Yorozuya (2006) carried out a study of parasitoids attacking mycophagous drosophilids. Collections were carried out in a fragmented forest in the Minami-osawa campus of Tokyo Metropolitan University and a wooded area at Takao in Tokyo, and in a grove of the Botanic Garden of Hokkaido University and a wooded area at Moiwa in Sapporo. At each site, three traps baited with different mushrooms were set; *Flammulina velutipes* (Curt.: Fr.), *Grifola frondosa* (Dicks.: Fr.) and *Agaricus bisporus* (Lange) were used as baits in Tokyo, while the first two and *Pleurotus cornucopiae* (Paulet) were used in Sapporo. All mushrooms were bought commercially. Mushrooms except *P. cornucopiae* were frozen before use, since freezing enhances decay; *Pleurotus cornucopiae* was used without freezing, since this mushroom decays rather promptly. A clump (about 30 g) of mushroom was placed in each trap, left for a week in the field, and brought back to the laboratory. At the time of collection of old mushrooms, new clumps were placed in traps. Since mushrooms

were left only for a week in the field, most drosophilid individuals oviposited in mushrooms remained as larvae at the time of collection. Therefore, pupal parasitoids were scarcely collected in this survey. Mushrooms collected from the field were placed in plastic containers with pieces of cloth. When drosophilid larvae in the baits pupated on cloth, they were collected, identified to species, and placed in separate Petri dishes. When flies or parasitoids emerged, they were identified and counted. Collections were carried out eight times (weeks) in May and June and nine times in September and October, 2009 in Tokyo, and nine times in July and August, 2010 in Sapporo. Voucher specimens were deposited in Graduate School of Environmental Earth Science, Hokkaido University.

Drosophilid species

Some closely related drosophilid species (e.g., *D. bizonata* Kikkawa & Peng and *D. orientacea* Grimaldi, James & Jaenike or members of the *quinaria* species group (*D. angularis* Okada, *D. brachynephros* Okada and *D. unispina* Okada)) cannot be discriminated by puparial morphology. The species of these pupae were determined by adult flies upon emergence.

Larval feeding niches (i.e., major breeding resources) of drosophilid species collected in this study were classified according to the studies of Kimura *et al*. (1977), Nishiharu (1980), Ichijô and Beppu (1990) and Mitsui *et al*. (2010) as follows: species mainly exploiting mushrooms (M), those exploiting decaying plant materials including mushrooms (P), those mainly exploiting fruits (F), and those mainly exploiting

decaying tree bark (T).

Parasitoid species

Parasitoids were identified to genus or species by adult morphology according to Yoshimoto (1962), Nordlander (1980), Belokobylskij (1998), Wharton (2002), Forshage and Nordlander (2008) and Novković *et al*. (2011). However, many parasitoids could not be identified to species by examining adult morphology. As a consequence, partial sequences of their mitochondrial COI (cytochrome oxidase subunit I) gene were determined for identification. DNA was extracted from whole body of morphologically different species by a modified phenol-chloroform protocol. Amplification of COI fragments was preformed mainly with a pair of primers (Folmer *et al*. 1994), LCO (5' -GGTCAACAAATCATAAAGATATTGG- 3') and HCO (5' -TAAACTTCAGGGTGACCAAAAAATCA- 3'). For species where the HCO-LCO primers failed to amplify, a pair of basal Hymenoptera COI forward (5' -ACNAATCAYAAANWTATTGG- 3') and basal Hymenoptera COI reverse (5' -TADACTTCHGGATGDCCAAARAATCA- 3') or a pair of basal Hymenoptera COI forward short (5' -CDTTYCCWCGWATAAATAATATAAG- 3') and HCO primers was used. The first two pairs amplified about 620bp, while the last did about 420bp. PCR reaction mixtures (24 μL) contained 1.24 μL $MgCl_2$, 0.5 μL dNTPs, 2.48 μL primers, 0.2 μL Ampli taq DNA polymerase, and 2.5 μL 10$\times$PCR buffer. Amplification was performed as follows: 10 min denaturation at 94 °C for one cycle; 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and 1.5 min elongation at 72 °C; 12

min final elongation at 72 °C for one cycle. All sequence reactions were done using the Big Dye Terminator Cycle Sequencing Kit (ABI) and sequencing was done with an ABI PRISM 3130 Genetic Analyzer. COI sequences were aligned manually, and nucleotide divergence was estimated by Kimura's two-parameter method (Kimura 1980a).

RESULTS

Drosophilid species

Table 1 shows the numbers of drosophilid species (pupae) collected at the two sites each in Tokyo and Sapporo. Data from different mushrooms were pooled, since the composition was not significantly different (data not shown). The composition differed considerably between Tokyo and Sapporo, but did not differ much between the two sites at each locality. In the present collections, *D. bizonata-D. orientacea* pupae were dominant in Tokyo and Sapporo (Table 1). In the samples of adult flies eclosed from the *D. bizonata-D. orientacea* pupae, *D. bizonata* comprised about 95% in Tokyo, and *D. orientacea* comprised 100% in Sapporo (data not shown). Since the *D. bizonata-D. orientacea* pupae comprised about 64% of total drosophilid pupae collected in Tokyo, *D. bizonata* was estimated to comprise about 61% of the total samples. On the other hand, *D. orientacea* comprised about 65% of the total samples in Sapporo. Other frequently recorded species were *D. immigrans* Sturtevant, *D. busckii* Coquilett, *D. curviceps* Okada & Kurokawa, *Scaptodrosophila coracina* Kikkawa & Peng, *D. lutescens* Okada in Tokyo, and *D. busckii*, *Sc. coracina* and *D. histrio* Meigen in Sapporo (Table 1).

Except for *D. histrio*, these species are not fungus specialists but generalists or fruit-feeders.

Parasitoid species

In the present survey, 13 species of parasitoids were discriminated, and nine of them were not identified to species. They belonged to four families, Braconidae (*Asobara japonica* Belokobylskij, *Phaenocarpa* sp. TK1, *Aphaereta* sp. TK1), Figitidae (*Ganaspis xanthopoda* (Ashmead), *Ganaspis* sp. TK1, *Ganaspis* sp. TK2, *Leptopilina heterotoma* (Thompson), *Leptopilina* sp. TK1, *Leptopilina* sp. TK2, *Kleidotoma* sp. TK1, *Leptolamina* sp. TK1), Diapriidae (*Trichopria* sp. TK1) and Pteromalidae (*Trichomalopsis microptera* (Lindeman)). Among them, *L. heterotoma* and *Leptopilina* sp. TK2 were morphologically very close.

To provide further information on the species discrimination, Braconidae and Figitidae species were compared for partial sequences of the COI gene (Table 2); sequences of eight species were determined in this study (376 bp for *Aphaereta* sp. TK1 and 585–690 bp for the others), and those of *A. japonica* from Tokyo and *L. heterotoma* from Sapporo and France were obtained from DDBJ (DNA Data Bank of Japan). In addition, two individuals cited as *L. heterotoma* Tokyo h2 and h3 in Novković *et al*. (2011) were included in comparison (their sequences were obtained from DDBJ), since they were morphologically close to *L. heterotoma* or *Leptopilina* sp. TK2. The nucleotide divergence between *Aphaereta* sp. TK1 and the other species was based on 319 bp, while that of the other pairs was based on 567 bp. No difference was observed

in the nucleotide sequence between *Phaenocarpa* sp. TK1 individuals from Tokyo and Sapporo, between *L. heterotoma* individuals from Sapporo and France, and between *L. heterotoma* h2 and *Leptopilina* sp. TK2. The nucleotide divergence between *L. heterotoma, Leptopilina* sp. TK2 (or *L. heterotoma* Tokyo h2) and *L. heterotoma* Tokyo h3 was relatively low (5.1–7.5%) and also between *Phaenocarpa* sp. TK1and *Aphaereta* sp. TK1 (8.9-9.2%). The other pairs showed much larger divergence (14.7-54.6%).

The numbers of parasitoids that emerged from drosophilid species in Tokyo and Sapporo were shown in Tables 3 and 4, respectively. In these Tables, drosophilid species from which no parasitoid emerged were not presented. At each locality, data on three mushroom species and the two sites were pooled, since the composition of parasitoid species did not differ significantly (data not shown). Among 10 Braconidae and Figitidae species collected in Tokyo, nine emerged from *D. bizonata* (Table 3). Among these nine species, *A. japonica* emerged from nine drosophilid species, *Phaenocarpa* sp. TK1 and *Kleidotoma* sp. TK1 from three, *Ganaspis* sp. TK1 and *Leptopilina* sp. TK2 from two, and the others only from *D. bizonata*. On the other hand, *G. xanthopoda* emerged only from *D. lutescens*. In Sapporo, *Phaenocarpa* sp. TK1 emerged only from *D. orientacea* and *L. heterotoma* from *D. orientacea*, *D. histrio* and *Sc. coracina* (Table 3).

In the present samples, the sex ratio was much biased in *A. japonica* (male/female=2/551), *Ganaspis* sp. TK2 (0/46) and *Leptopilina* sp. TK1 (1/350), suggesting that they reproduce parthenogenetically. In the other abundant and common parasitoids, the male/female ratio ranged from 0.3 to 0.7.

DISCUSSION

Drosophila orientacea comprised about 65 % of drosophilid flies that emerged from mushroom baits in the survey in Sapporo (the cool-temperate region). However, this species comprised only 1-32 % of drosophilids breeding on naturally-occurring mushrooms in Sapporo and adjacent localities (Kimura *et al*. 1977; Kimura 1980b; Kimura & Toda 1989; Toda *et al*. 1999; Yorozuya 2006). The abundantly collected species from naturally-occurring mushrooms in Sapporo are *Hirtodrosophila sexvittata* Okada, *H. ussurica* Duda, *H. trivittata* Strobl and *H. trilineata* Chung which were scarcely collected in this survey, most likely because mushrooms used as baits in the present collections were decayed and did not attract these *Hirtodrosophila* species that prefer fresh mushrooms (Kimura 1980b; Kimura & Toda 1989). In the survey in Tokyo (the warm-temperate region), *D. bizonata* comprised about 60 % of drosophilid flies. This species also comprises more than 50 % of drosophilid flies breeding on naturally-occurring mushrooms in Tama Forest Science Garden located close to the Takao site (Nishiharu 1980; Takahashi *et al*. 2005). The dominance of *D. bizonata* even in naturally-occurring habitats would be related to the rarity of *Hirtodrosophila* species in the warm-temperate region (Nishiharu 1980; Takahashi *et al*. 2005).

In the present study, 13 species of parasitoids are discriminated; three belong to Braconidae (the genera *Asobara, Aphaereta* and *Phaenocarpa*), eight to Figitidae (*Ganaspis, Leptopilina, Kleidotoma* and *Leptolamina*), one to Diapriidae (*Trichopria*), and one to Pteromalidae (*Trichomalopsis*). Braconidae and Figitidae species are larval

parasitoids, while Diapriidae and Pteromalidae species are pupal parasitoids (Carton *et al*. 1986). In these species, *Leptopilina* sp. TK2 has an identical sequence (567 bp) of the COI gene with *L. heterotoma* Tokyo h2 in Novković *et al*. (2011), suggesting that they are conspecific. On the other hand, *L. heterotoma*, *Leptopilina* sp. TK2 (i.e., *L. heterotoma* Tokyo h2) and *L. heterotoma* Tokyo h3 of Novković *et al*. (2011) showed substantial differences (5.1–7.5%) in the COI sequence, but there is no difference between Sapporo and French individuals of *L. heterotoma* and between two individuals of *Leptopilina* sp. TK2 from Tokyo. They may belong to different to species, although Novković *et al*. (2011) tentatively treated them as variation. Further study is needed to determine their species status. The other species pairs show rather large difference in the nucleotide sequence of the COI gene, supporting that they belong to different species.

Among the 11 larval parasitoid species, *A. japonica*, *L. heterotoma*, *G. xanthopoda* and *G*. sp. TK1 were also reported to parasitize drosophilid flies breeding on fruits (Janssen et al. 1988; Mitsui *et al*. 2007; Ideo *et al*. 2008; Mitsui & Kimura 2010: *Ganaspis* sp. TK1 was cited as *Ganaspis* sp. 2 in Mitsui *et al*. (2007)), revealing their wide habitat use (i.e., they search hosts on both mushrooms and fruits). Among these species, *A. japonica* and *L. heterotoma* parasitize various drosophilid species including frugivorous and mycophagous ones (Janssen et al. 1988; Mitsui *et al*. 2007; Ideo *et al*. 2008; Mitsui & Kimura 2010). On the other hand, *Ganaspis* sp. TK1 has been recorded mostly from *Sc. coracina* which breeds on both fruits and mushrooms (Mitsui et al. 2007). In this study, *G. xanthopoda* was recorded only from *D. lutescens*. However, fruits, not fungi, are the main larval resources of *D. lutescens*, and its larvae

in fruits (banana) are often heavily parasitized by *G. xanthopoda* (Mitsui & Kimura 2010). Thus, the habitat use of these four parasitoid species seems to reflect the occurrence (i.e. resource use) of their suitable hosts.

In Tokyo, almost all parasitoid species emerged from *D. bizonata-D. orientacea* pupae. It is not certain whether these parasitoids attack both of them or specialize either of them, since these two host species cannot be discriminated at the pupal stage. However, it can be said that most parasitoids attacked *D. bizonata*, the most abundant mycophagous drosophilid in Tokyo, since it comprised about 95% of the *D. bizonata-D. orientacea* pupae. In the Netherlands, Driessen *et al*. (1990) also observed that many parasitoids attacked the most abundant drosophilid species, *D. phalerata* Meigen. In addition, Yorozuya (2006) observed in the mycophagous drosophilid community in Tomakomai that more abundant species were more frequently parasitized. Thus, the abundance of potential hosts is one of the important factors affecting the host use of parasitoids.

In this study, two species belonging to the *immigrans* species group, *D. immigrans* and *D. curviceps*, were rarely parasitized, although they were rather abundant. In addition, *D. immigrans* larvae breeding on fruits are also rarely parasitized (Carton *et al*. 1986; Mitsui *et al*. 2007; Ideo *et al*. 2008; Mitsui & Kimura 2010). It is not known why they are rarely parasitized, but Van Alphen and Janssen (1982) suggested that *D. immigrans* larvae had thick cuticle that could present the insertion of parasitoid ovipositors.

Among the 10 parasitoids recorded in Tokyo, only three, *A. japonica*,

Phaenocarpa sp. TK1 and *Leptopilina* sp. TK1, occur in Sapporo or Tomakomai (Yorozuya 2006; Mitsui *et al*. 2007) (samples cited as *Ganaspis* sp. in the Yorozuya's paper included two different species, *Ganaspis* sp. and *Leptopilina* sp. TK1). Particularly, Figitidae species attacking mycophagous drosophilids are richer in Tokyo; eight species were collected from Tokyo in this study, whereas only three species were recorded from Sapporo and Tomakomai (Yorozuya 2006). In contrast, the number of Braconidae species does not so much differ between these regions; three from Tokyo and four from Sapporo and Tomakomai. It is not known why the species diversity of Figitidae differs between Tokyo and Sapporo or Tomakomai.

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Table 1. Number of drosophilid pupae collected at two sites each in Tokyo and Sapporo, with information on their major breeding resources: M (mushrooms), F (fruits), P (decayed plant materials including mushrooms), and T (tree sap and bark).

	Tokyo			Sapporo			Total	Main
	Minami-osawa	Takao	Total	Botanic Garden	Moiwa	Total		breeding resources
Drosophila bizonata Kikkawa & Peng	6160*	5714*	11874*			0	11874*	M
D. orientacea Grimaldi, James & Jaenike				1312	2554	3866	3866	M
D. immigrans Sturtevant	1556	1027	2583	85	2	87	2670	F
D. busckii Coquilett	1074	556	1630	613	321	934	2564	P
Scaptodrosophila coracina Kikkawa & Peng	395	172	567	247	181	428	995	M & F
D. curviceps Okada & Kurokawa	195	441	636			0	636	P
D. histrio Meigen	109	14	123	71	332	403	526	M
D. lutescens Okada	61	329	390			0	390	F
D. angularis Okada	166	57	223			0	223	M
Styloptera nishiharui Okada	134	15	149			0	149	M
Hirtodrosophila fascipennis (Okada)		108	108			0	108	M
D. nigromaculata Kikkawa & Peng			0	8	73	81	81	P
D. lacertosa Okada			0	7	47	54	54	T

D. rufa Kikkawa & Peng	20	32	52			0	52	F
D. sternopleuralis Okada & Kurokawa	17	20	37			0	37	F
D. annulipes Duda	4	29	33			0	33	P
D. unispina Okada		24	24			0	24	M
Leucophenga quinquemaculipennis Okada			0		22	22	22	M
H. histrioides Okada & Kurokawa			0	13	5	18	18	M
Others	5	6	11	13	7	20	31	

*Including *D. orientacea*.

Table 2. Nucleotide divergence (%) between Brachonidae and Figitidae species based on partial sequences of the COI gene.

		Accession number	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*A. japonica* (Tokyo)*	AB456699													
2	*Phaenocarpa* sp. TK1 (Tokyo)	AB624296	17.4												
3	*Phaenocapra* sp. TK1(Sapporo)	AB624297	17.0	0.3											
4	*Aphaereta* sp. TK1	AB624306	17.8	9.2	8.9										
5	*Leptopilina heterotoma* (France)*	AB456712	39.6	33.5	33.0	30.2									
6	*L. heterotoma* (Sapporo)*	AB583568	39.6	33.5	33.0	30.2	0.0								
7	*L. heterotoma* h2*	AB583574	43.4	36.0	35.5	35.0	7.5	7.5							
8	*Leptopilina* sp. TK2	AB624303	43.4	36.0	35.5	35.0	7.5	7.5	0.0						
9	*L. heterotoma* h3*	AB583575	41.7	37.0	36.5	35.5	6.9	6.9	5.1	5.1					
10	*Leptopilina* sp. TK1	AB624302	40.1	42.2	41.7	35.0	15.9	15.9	16.2	16.2	15.1				
11	*Ganaspis xanthopoda*	AB624301	46.8	41.2	41.7	38.6	25.7	25.7	27.0	26.6	24.8	27.0			
12	*Ganaspis* sp. TK1	AB624299	47.9	40.6	41.2	40.6	21.4	21.4	22.3	21.9	22.7	22.3	15.1		
13	*Ganaspis* sp. TK2	AB624300	46.8	45.6	45.1	40.7	27.4	27.4	28.4	27.5	26.6	28.4	15.9	14.7	
14	*Kleidotoma* sp. TK1	AB624304	54.6	48.3	47.6	44.6	26.3	26.3	28.2	26.3	26.7	28.2	28.5	24.4	24.0

* Nucleotide sequences were obtained from DDBJ.

Table 2. Numbers of flies and parasitoids that emerged from drosophilid pupae collected in Tokyo. Aj (*Asobara japonica*), Ph (*Phaenocarpa* sp. TK1), Ap (*Aphaereta* sp. TK1), Kl (*Kleidotoma* sp. TK1), G1 (*Ganaspis* sp. TK1), G2 (*G.* sp. TK2), Gx (*G. xanthopoda*), L1 (*Leptopilina* sp. TK1), L2 (*L.* sp. TK2), Ll (*Leptolamina* sp. TK1), Dia and Tr (Diapriidae: *Trichopria* sp. TK1), un (undetermined: wasps escaped from Petri dished at collection).

	No. of pupae collected	Flies	Parasitoids												The rate of total parasitism (%)	Neither fly or wasp emerged
			Braconidae			Figitidae							Dia			
			Aj	Ph	Ap	Kl	G1	G2	Gx	L1	L2	Ll	Tr	un		
*D. bizonata**	11874	7847	553	560	2	354	1	46		351	32	1		60	20.0	2067
D. immigrans	2583	2365	1										2		0.1	215
D. busckii	1630	1222	216										5		15.3	187
D. curviceps	636	588	8								1				1.5	39
Sc. coracina	567	250	23				13							2	13.2	279
D. lutescens	390	302	5			1			42						13.7	40
D. angularis	223	150	14	2		3									11.2	54
St. nishiharui	149	139	1												0.7	9
D. histrio	123	101	2												1.9	20
D. sternopleuralis	37	34		1											2.9	2

**D. orientacea* is included.

Table 4. Numbers of flies and wasps emerged from drosophilid pupae collected in Sapporo. Ph: *Phaenocarpa* sp. TK1, Lh: *Leptopilina heterotoma*, Tm: *Tricholopalopsis microptera*.

	No. of pupae collected	Fly	Parasitoids			The rate of total parasitism (%)	Neither fly or wasp emerged
			Ph (Braco.)	Lh (Figit.)	Tm (Ptero.)		
D. orientacea	3866	1588	380	4	1	19.5	1893
Sc. coracina	428	206		1		0.5	221
D. histrio	403	185		1		0.5	217
D. histrioides	18	7			1	12.5	10