Distribution and population structure of two phylogroups of the parasitoid Encarsia smithi (Hymenoptera: Aphelinidae) in tea fields infested with the invasive camellia spiny whitefly Aleurocanthus camelliae (Hemiptera: Aleyrodidae) in Shizuoka Prefecture, Japan

Applied Entomology and Zoology

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Abstract:	A recent study revealed that two phylogenetic groups of the parasitoid Encarsia smithi (Hymenoptera: Aphelinidae) can attack the camellia spiny whitefly Aleurocanthus camelliae (Hemiptera: Aleyrodidae), an invasive pest of Japanese tea fields. Type I was introduced in 1925 from China to Japanese citrus orchards to control the citrus spiny whitefly A. spiniferus, but it has also recently appeared in several tea fields. Type II, presumably introduced accidentally, was also found in many tea fields. However, little is known about distribution and their relative importance as a biocontrol agent in tea fields. To investigate these aspects, we developed specific PCR for the two groups using a variation in their nuclear ribosomal DNA's ITS region. We then surveyed their distribution in 23 tea fields in Shizuoka Prefecture, Japan, from 2013 to 2015 using this specific PCR. We found that both types were distributed, sometimes coexisting, in many tea fields during 2013 - 2015, although the population structure of these types varied with the field, year and season. These results suggest that A. camelliae can be controlled unintentionally by accidentally-introduced exotic natural enemies (Type II) and/or Type I species originally introduced to control other invasive pests such as A. spiniferus.
Response to Reviewers:	Dear Dr. Kainoh, We are most grateful to you and the reviewers for your helpful comments on our revised manuscript. We accepted all the advice and have carried out the corrections suggested. We trust that the revised version of our paper is now suitable for publication in Applied Entomology and Zoology.

	Gratefully, Kaori Yara Reviewers' comments: I have only a few comments. I also used the "track change in Word" to indicate the style corrections. Please download the file. Response: We have corrected the style of our manuscript according to the "track change in Word". Table 3: Numbers preceding name of city or town correspond to those in Figure 2. ->> Numbers preceding name of city or town correspond to those in Figure 3. Response: We have corrected the revised Table 3. We hope our responses are satisfactory. We look forward to your consideration. With many thanks, Kaori Yara

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Abstract

A recent study revealed that two phylogenetic groups of the parasitoid *Encarsia smithi* (Hymenoptera: Aphelinidae) can attack the camellia spiny whitefly *Aleurocanthus camelliae* (Hemiptera: Aleyrodidae), an invasive pest of Japanese tea fields. Type I was introduced in 1925 from China to Japanese citrus orchards to control the citrus spiny whitefly *A. spiniferus*, but it has also recently appeared in several tea fields. Type II, presumably introduced accidentally, was also found in many tea fields. However, little is known about distribution and their relative importance as a biocontrol agent in tea fields. To investigate these aspects, we developed specific PCR for the two groups using a variation in their nuclear ribosomal DNA's ITS region. We then surveyed their distribution in 23 tea fields in Shizuoka Prefecture, Japan, from 2013 to 2015 using this specific PCR. We found that both types were distributed, sometimes coexisting, in many tea fields during 2013–2015, although the population structure of these types varied with the field, year and season. These results suggest that *A. camelliae* can be controlled unintentionally by accidentally-introduced exotic natural enemies (Type II) and/or Type I species originally introduced to control other invasive pests such as *A. spiniferus*.

Keywords

Aleurocanthus camelliae · *Encarsia smithi* · parasitoid wasp · phylogenetic group · specific PCR

Introduction

Biological control is an effective means of controlling agricultural pests (Cock et al. 2016; van Lenteren 2012; Van Driesche et al. 2010). Exotic natural enemies are often employed to control invasive pests (Hajek et al. 2016). Many studies have demonstrated successful control of invasive pests by intentionally-introduced biocontrol agents (Bellows 2001; Cock et al. 2016). However, fewer studies have documented the unintentional suppression of invasive pests by accidentally-introduced natural enemies or species already introduced to control other invasive pests (Hajek et al. 2016; Kenis et al. 2017). This paper focuses on the unexpected impact of exotic enemies on invasive pests observed in Japanese tea fields.

Encarsia smithi (Silvestri) (Hymenoptera: Aphelinidae) is a well-known parasitoid of the citrus spiny whitefly *Aleurocanthus spiniferus* (Quaintance) and *A. woglumi* Ashby (Hemiptera: Aleyrodidae), both citrus pest whiteflies, in many countries (Nguyen and Sailer 1987). In Japan, *E. smithi* was first introduced from southern China to Nagasaki Prefecture in 1925 to control an *A. spiniferus* invasion in a citrus orchard (Kuwana 1934). The parasitoid was then mass-released in other areas from 1961 to 1998 as part of a government project. These intermittent releases resulted in a drastic decrease in *A. spiniferus* populations in most areas, except for the occasional occurrence in limited regions (Ohgushi 1969). This classical biological control is one of the best-known and most successful ones in Japan (van den Berg and Greenland 1997).

Interestingly, *E. smithi* has recently reappeared in many tea fields as a significant natural enemy of the invasive camellia spiny whitefly *A. camelliae* Kanmiya and Kasai, the occurrence of which was first reported in Japan in 2004 (Yamashita et al. 2016). Uesugi et al. (2016a) reported, using mtDNA COI and microsatellite variability, that the

E. smithi samples collected from nine tea fields comprised two phylogenetic groups: Type I was observed in two fields (in Shizuoka and Fukuoka Prefectures), while Type II was identified in another seven fields (in Shizuoka, Gifu, Mie, Shiga, Kyoto, Nara and Hyogo Prefectures). They also reported that all the *E. smithi* individuals collected from ten citrus orchards were Type I. It therefore appears that Type I is derived from the populations released for controlling *A. spiniferus*, while Type II might have been unintentionally introduced to tea plantations alongside the invasion of *A. camelliae* (Uesugi et al. 2016a).

Although each type of *E. smithi* can parasitize *A. camelliae* in tea fields, their relative importance as biological control agents remains to be evaluated. Whether Type I frequently predominates over Type II in tea fields is particularly open to question. The ecological characteristics of each type have also not been closely studied. For example, it is unclear whether they tend to be distributed separately in different tea fields or coexist in the same tea fields; the results of Uesugi et al. (2016a) may suggest the former possibility. Further, their intraspecific interaction (e.g., competition or hybridization) has never been examined. To clarify these matters, we need to develop a new molecular method of identifying their hybrids as well as each group.

In this study, we first developed PCR specific to the two phylogroups by using a variation in the ITS region of their nuclear ribosomal DNA. Thereafter, by using this specific PCR, we surveyed the population structures of the types in tea fields in Shizuoka Prefecture over three years. The relative importance of these types as a natural enemy and their complex ecological interactions in tea fields and citrus plants were investigated.

Materials and methods

Developing specific PCR

Encarsia smithi, listed in Table 1, were used to sequence the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (nrDNA) region and to a develop specific PCR to distinguish the two phylogroups. They were already part of the samples taken by Uesugi et al. (2016a) and their phylogroups had been already been identified. DNA was extracted from these individuals with a DNeasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. The DNA was eluted with 50 µl of Buffer AE from the kit. The DNA sequences of the ITS region were determined according to Schmidt et al. (2006) with some modifications. To amplify the ITS region, two primers, TW81 5'-GTTTCCGTAGGTGAACCTGC-3' and Aed5.8R 5'-GAGAACAGCAGGAACACAGAAC-3' (Brust et al. 1998) were used. PCR was carried out in 25-µl reaction mixtures containing 0.2 mM each of dNTP, 0.2 µM of each primer, 1.0 µl template DNA, 0.65 U of *TaKaRa Ex Taq* (Takara Bio Inc.), and 1× *Ex Taq* buffer (2.0 mM Mg^{2+} concentration). The cycling conditions were as follows: 94 °C for 5 min, 40 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, and 72 °C for 5 min in a GeneAmp PCR System 9700 (Perkin-Elmer). PCR products were purified using ExoSAP-IT (Affymetrix), and both strands were directly sequenced using the same primers as in the PCR and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions.

From the ITS sequence data obtained (DDBJ/EMBL/GenBank Acc# LC326520, LC326521), we designed four specific primers that included specific InDel regions: ITS1-EsI-F, ITS2-EsI-R, ITS1-EsII-F, and ITS2-EsII-R (Table 2). Specific PCR using

the designed primers was carried out under almost the same conditions as described above, but the cycling conditions were as follows: 96 ˚C for 1 min, 40 cycles at 94 ˚C for 1 min, 57 ˚C for 1 min, 72 ˚C for 1.5 min, and 72 ˚C for 7 min on a MyCycler (Bio-Rad Laboratories, Inc.). The PCR products were electrophoresed in 2% agarose gel in 1×TAE buffer at 100 V for 30 min and visualized by staining with GelRed Nucleic Acid Gel Stain (Biotium).

The designed primers' specificity was validated using the same specimens that were used to determine the ITS sequences (Table 1). Their usability in this study was confirmed by using specimens that had been taken from the same population in Table 1 but with their DNA extracted with PrepMan Ultra Reagent (Applied Biosystems) as described below.

Sample collection

Encarsia smithi were collected from 24 tea fields in Shizuoka Prefecture, Japan, from 2013 to 2015 (Fig. 1, Table 3). Leaves of the tea plant *Camellia sinensis* infested with the host, camellia spiny whitefly *Aleurocanthus camelliae* nymphs, were collected from April to August of each year (Table 3), placed in plastic boxes, and kept in an incubation room (23 ˚C, 15L 9D). Adult *E. smithi* that emerged from the hosts were collected and stored in 99.5 % ethanol at 4 ˚C until genetic analysis.

One *E. smithi* adult that emerged from another host, the citrus spiny whitefly *A. spiniferus* infesting a citrus leaf, was also used (Table 3). It was obtained in a similar manner to that described above: a citrus leaf infested with *A. spiniferus* was collected in August of 2014. The citrus tree from which the leaf was collected was surrounded by tea fields.

Identification by type-specific PCR

DNA was extracted from single, whole specimens using PrepMan Ultra Reagent (Applied Biosystems) according to the manufacturer's instructions, not homogenizing but instead vortexing the body. 30 µl of reagent was used per individual. For each individual, two kinds of specific PCR, using two primers (ITS1-EsI-F and ITS2-EsI-R) specific to Type I, and using the other two primers (ITS1-EsII-F and ITS2-EsII-R) specific to Type II (Table 2), were carried out. The PCR and electrophoretic conditions were the same as described above.

The specimens were identified as Type I when they produced the expected amplicon (ca. 993 bp band) by PCR specific to Type I only; the specimens were identified as Type II when they produced the expected amplicon (ca. 890 bp band) by PCR specific to Type II only; and the specimens were identified as hybrids when they produced the expected amplicons by both the specific PCRs.

The variation in frequency of genetic types of *E. smithi* for different dates of collection was tested using the generalized linear model (GLM) with a binomial distribution at each sampling location using R (version 3.4.0) statistical software. The likelihood ratio test was performed using "car" package versions 2.1 - 6 in R version 3.4.0. In the model, data from hybrid genotypes was not used.

Results

Specific PCR

We obtained approximately 1000-bp sequences containing 18S rDNA, ITS1, 5.8S rDNA, and ITS2 region (Acc# LC326520, LC326521). Sufficient sequence data was obtained to design specific primers to two phylogroups by inspecting electropherograms and by distinguishing between intra- and inter-phylogroup variation. The specific

primers we designed produced the expected amplicons in each of two specific PCRs (Fig. 2). When two phylogroups' DNAs were mixed at a ratio of 1 : 1 and used as template DNA, both amplicons were produced (Fig. 2). Consistent results were obtained when we used DNA templates prepared using PrepMan Ultra Reagent. Specific PCR was therefore conducted to determine the phylogroup for each *E. smithi* and the hybrid between them.

Occurrence of the two phylogroups and their hybrid

The specific PCR revealed two types of *E. smithi* and their F_1 hybrid in this study. The GLM suggests that the frequency of the two types changed significantly with different collection dates in 10 locations (Fujinomiya 3-4, Shizuoka 2-3, Fujieda, Shimada 1-2, Hamamatsu 1, 3-4; $p < 0.05$, likelihood ratio test: Fig. 3). However, no general patterns in population structure changes were observed in these fields. For example, all individuals collected from Shimada 1 in 2013 and 2015 were identified as Type I and Type II, respectively. Type I predominated over Type II at Shizuoka 3 and Fujieda in 2013, while the opposite pattern was observed in each of these fields in 2015. Type II predominated over Type I at Hamamatsu 1 and 4 in 2013, and all individuals from Hamamatsu 4 in 2014 were Type II; but Type I predominated over Type II in each field in 2015. At Fujinomiya 3 and Hamamatsu 3, Type II predominated over Type I in 2013, while all specimens from this field in 2015 were identified as Type I.

The GLM suggests that frequency of the two types did not change significantly among different collection dates in the other nine locations (Numazu 1 and 2, Fujinomiya 1 and 2, Shizuoka 1 and 4, Makinohara, Kawanehoncho and Hamamatsu 2; $p > 0.05$, likelihood ratio test: Fig. 3). For example, all individuals collected from

Fujinomiya 1 and 2 and Shizuoka in 2013 and 2015 were identified as Type I. This was also the case for parasitoids collected from Makinohara in 2013 and the spring of 2014. The dominant type in Numazu 2 was Type I in 2013 and 2015. On the other hand, Type II predominated over Type I at Kawanehoncho in 2013, 2014 (summer) and 2015.

In this study, a total of 491 parasitoids were collected in the tea fields over the three years, of which 277 and 208 individuals (56.4 % and 42.4 %) were respectively identified as Type I and Type II. Only six individuals that remained (1.2 %) were identified as their F_1 hybrids. Type I was observed in all the 23 tea fields investigated in 2013 (100 %), while Type II was observed in 12 fields in the same year (50.2 %) (Fig. 3). We also found Type I in 14 fields (77.8 %), and Type II in 10 fields (55.6 %), out of 18 fields investigated in 2015.

Although we did search for *E. smithi* on citrus leaves infested with *A. spiniferus*, only one parasitoid was eventually collected from this plant-host combination. Interestingly, this individual was identified as Type II.

Discussion

In Shizuoka Prefecture, the invasive pest *A. camelliae* was first found in a tea field in Kikugawa (Kurasawa) in October 2010; the species then rapidly expanded its distribution throughout the prefecture in 2011–2012 (Ozawa et al. 2015). Ozawa et al. (2015) also found *E. smithi* in most of the tea fields they investigated (105 of 121 fields) located in various regions in the prefecture from December 2012 to March 2013, such as Numazu, Fujinomiya, Shizuoka, Shimada, Makinohara and Hamamatsu. We also collected *E. smithi* from the same regions or local areas and then found that 1) both types were distributed, sometimes coexisting, in many tea fields in Shizuoka Prefecture

in 2013–2015; and 2) one type sometimes predominated over the other in these fields. There are a few field studies suggesting that parasitism by *E. smithi* (type unknown) affected the density of *A. camelliae* in tea fields in Shizuoka Prefecture (Ozawa et al. 2015; Uesugi et al. 2016b). From these results and previous findings, we can say that each type of *E. smithi* played a role in controlling *A. camelliae* in the tea fields investigated. Further, to the best of our knowledge, this is the first report to show that populations of an invasive pest (*A. camelliae*) can be controlled unintentionally by accidentally-introduced exotic enemies (Type II) and/or exotic but already established species used as control agents against other invasive pests (Type I and *A. spiniferus*, respectively).

Interestingly, Type I was already present in all of the 23 tea fields investigated up until 2013, whereas Type II had been distributed in half of these fields until that year (Fig. 3). A plausible explanation for the more frequent observation of Type I is that they were endemic to the regions investigated. In Shizuoka Prefecture, both citrus and tea are intensively cultivated, sometimes close to each other, in the same regions. It is likely that Type I parasitoids that had inhabited citrus trees with *A. spiniferus* had dispersed to neighboring tea fields to exploit *A. camelliae* as a new host. After Type I had established populations in the tea fields, they might have rapidly dispersed to other tea fields in the same local areas to exploit the abundant available hosts. It appears that *E. smithi* (type unknown) in tea fields has rapidly expanded its distribution via frequent aerial dispersal between tea fields (Uesugi et al. 2016b). Further studies are needed to clarify the expansion of distribution of each type and its underlying mechanisms, as well as the consequences of biological pest control applied using these types in tea fields.

We found that the two types of parasitoids sometimes coexisted in the same tea

fields (Fig. 3). In our previous study (Uesugi et al. 2016a), they were observed allopatrically in tea fields. In this study, in contrast, both types were sympatrically found in 12 of 23 tea fields in 2013, and 6 of 18 fields in 2015 (Fig. 3). This prompts the interesting question as to whether there was an intense competitive interaction between the two types of parasitoids, causing the displacement of one type by the other. One example of this is the displacement of *Torymus beneficus* (Hymenoptera: Torymidae) by *T. sinensis*, a simultaneously indigenous and introduced parasitoid of the chestnut gall wasp *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae), in Japanese chestnut plantations (Yara et al. 2007, 2010). In this study, however, no fiercely competitive interaction was found between them, and no general pattern of change in their population structure. The only evidence of any interaction was hybridization, although it was detected only rarely (Fig. 3). The influence of hybridization and other possible interactions between the two parasitoids on the outcome of the biological control remains to be evaluated in further studies.

This is the first report to show that Type II can inhabit the vegetation surrounding tea fields. In our previous study, Type II was collected from tea plants only, whereas Type I was collected from citrus plants, (e.g., *Citrus unshiu*) as well as from tea plants (Uesugi et al. 2016a). Because *A. spiniferus* is generally distributed at low density in citrus plants, we were able to obtain only one parasitoid, identified as Type II, from a citrus tree infested with *A. spiniferus* beside a tea field. We conclude that each type of *E. smithi* can exploit both tea plants and the surrounding vegetation infested with different host species.

Our findings in this study suggest complex interactions among the parasitoids, whiteflies and host plants. Previous studies have focused on simple interactions between

E. smithi (mainly Type II) and *A. camelliae* as a pest of tea (Ozawa et al. 2015; Uesugi et al. 2016b) or between *E. smithi* (Type I) and *A. spiniferus* as a citrus pest (Kawamura 1976) (Fig. 4a). Other than this, our study makes clear the importance of Type I, dispersing from surrounding vegetation, as a biocontrol against *A. camelliae* (Fig. 4b). Our results also suggest the possibility of intraspecific interactions between the two types of parasitoids in tea fields and the surrounding vegetation (e.g., citrus), although their influence on the biological control of the two whiteflies remains to be evaluated. Further field and laboratory studies should aim to explore these complicated interactions in tea fields and the surrounding vegetation and to clarify the ecological characteristics of these parasitoids. In a previous study, we found that Type I and Type II have different ecological characteristics in terms of number of offspring and emergence patterns, when rearing them using citrus seedlings infested with *A. spiniferus* (Yara et al. 2017). This pattern may be also seen in their relationship with *A. camelliae*, although we need a rearing method for the parasitoids using this host.

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Figure legends

Fig. 1 A map of the Japanese Archipelago. Shizuoka Prefecture, the study region in this article, is circled.

Fig. 2 PCR amplification with the specific primer pairs for each phylogroup of *Encarsia smithi*. a: primers specific to Type I used; b: primers specific to Type II used. Lanes 1 - 2: Type I; 3 - 4: Type II; 5-6: DNAs of Type I and II were mixed at a ratio of 1 : 1 and used as template DNA. M: 100 bp ladder marker.

Fig. 3 Population structure of the two phylogroups of *Encarsia smithi* and their hybrids in tea fields in Shizuoka Prefecture in 2013–2015. The numbers in the small circles on the map, which represent the sampling sites, correspond to those tabulated in Table 3. The numbers in the pie charts indicate the numbers of parasitoids (Type I, Type II and their hybrids) found at each sampling site. Asterisks indicate significant differences among different years of collection ($p < 0.05$, likelihood ratio test).

Fig. 4 A schematic illustration of the observed interactions among parasitoids, host whiteflies, and host plants in (a) previous studies and (b) the present study. Thick arrows show frequently observed interactions or events, and thin arrows show less frequently observed interactions or events.

Figure 1

Fig.1

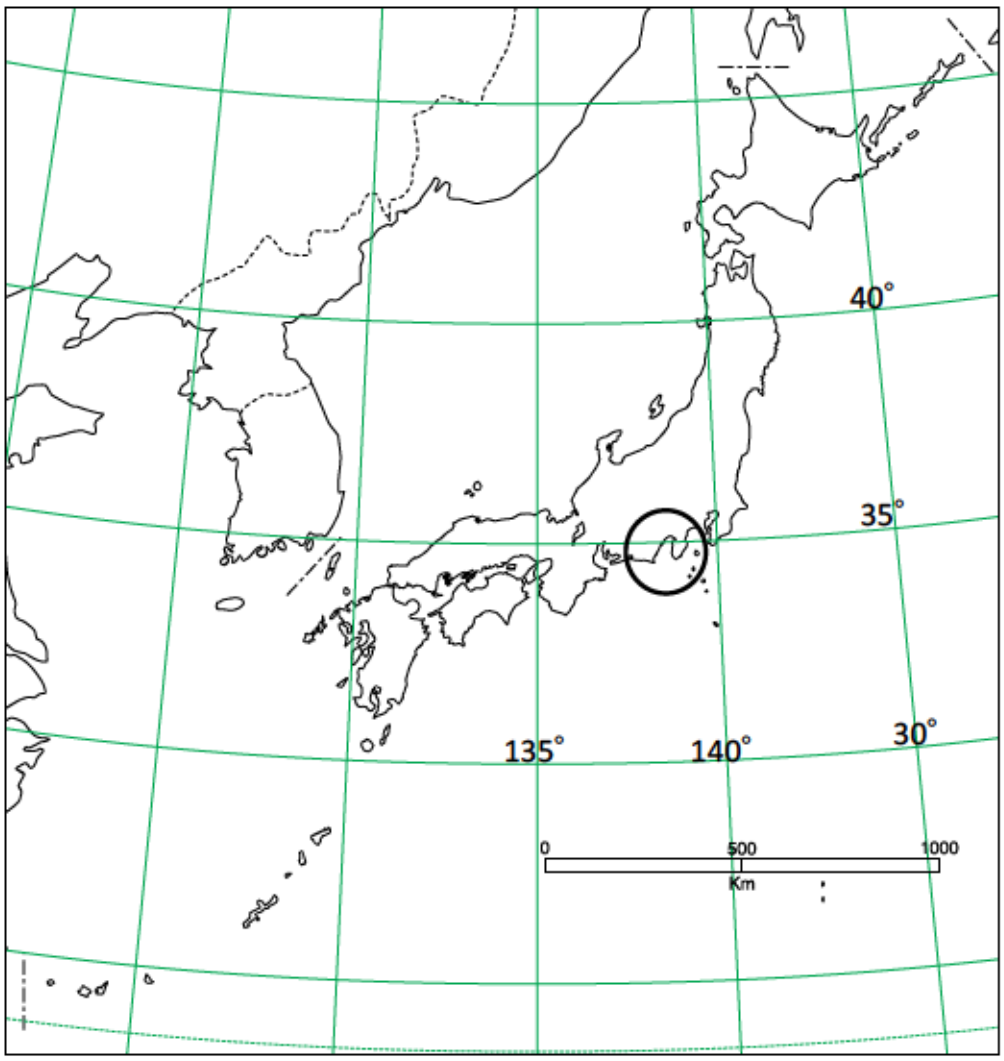

Figure 2

Fig. 2

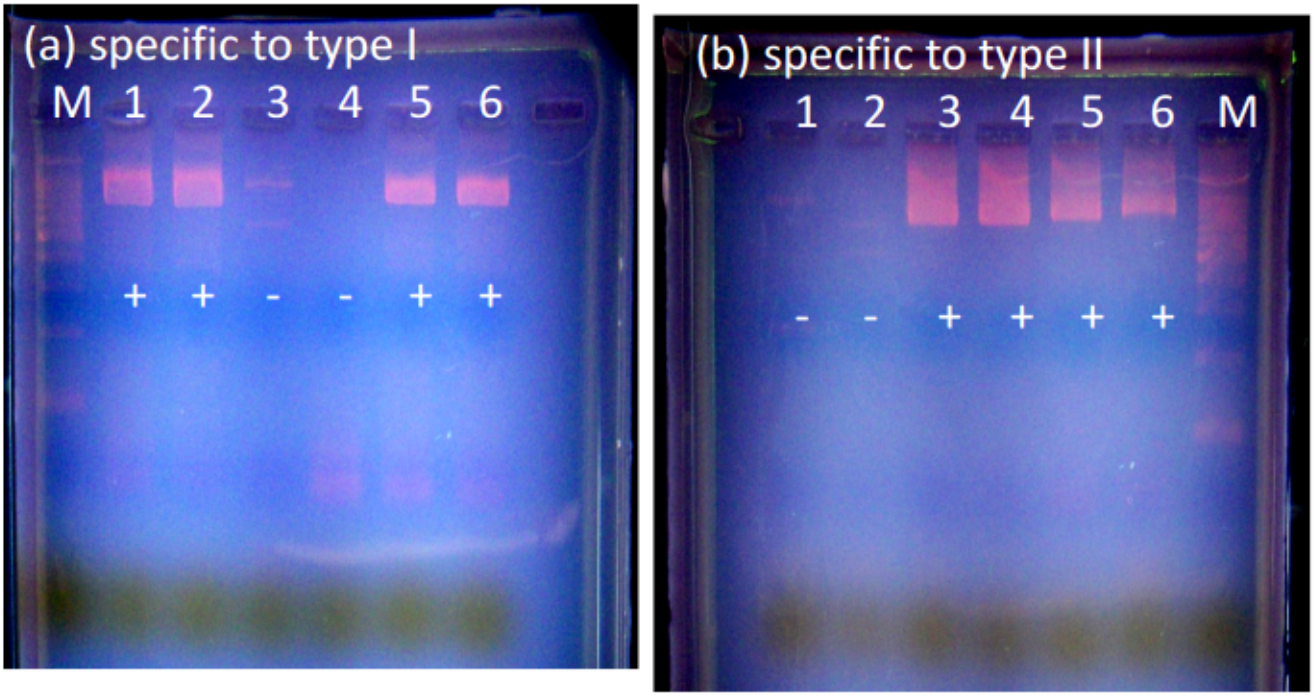

Figure 3

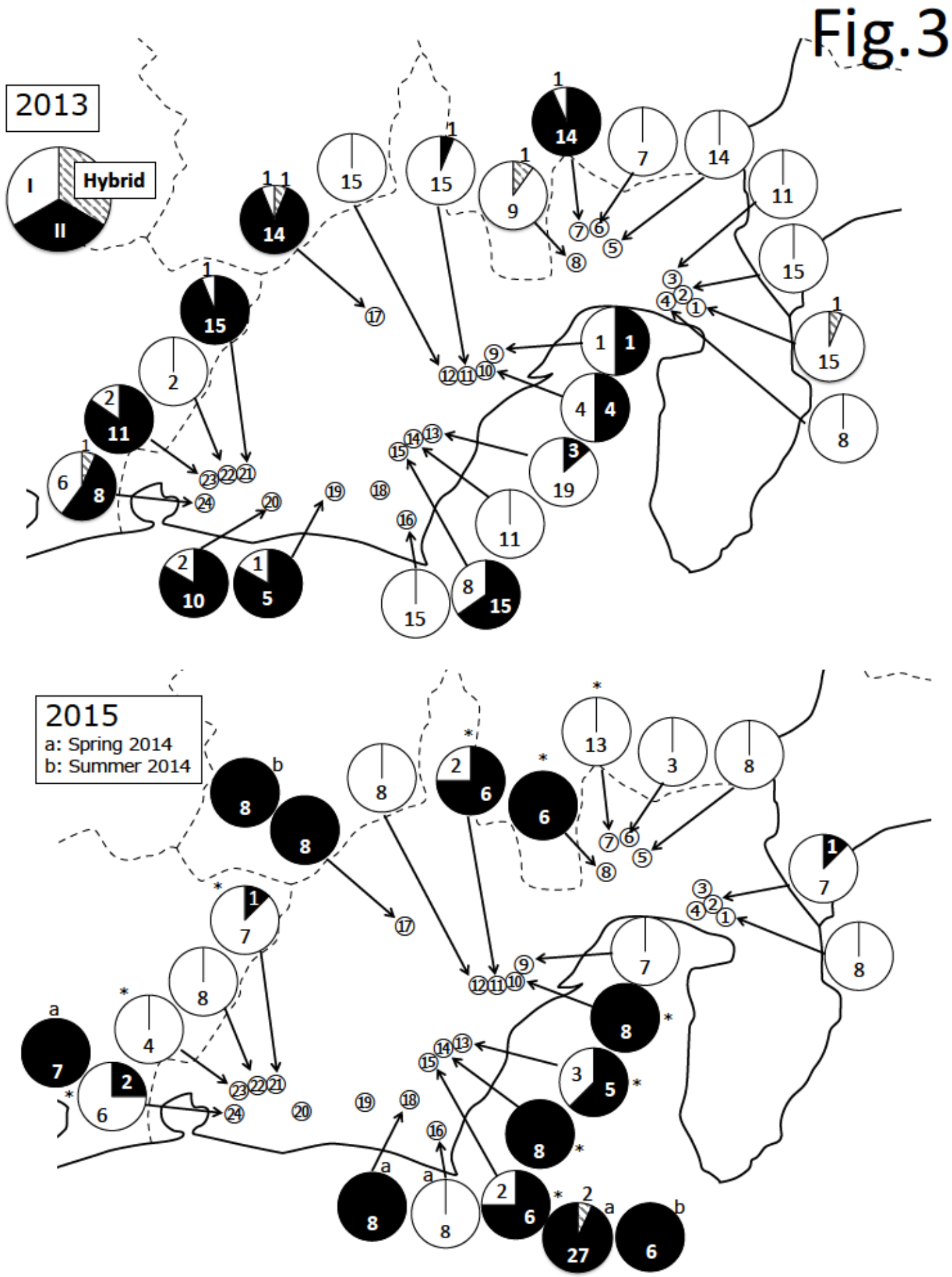

Figure 4

Fig.4

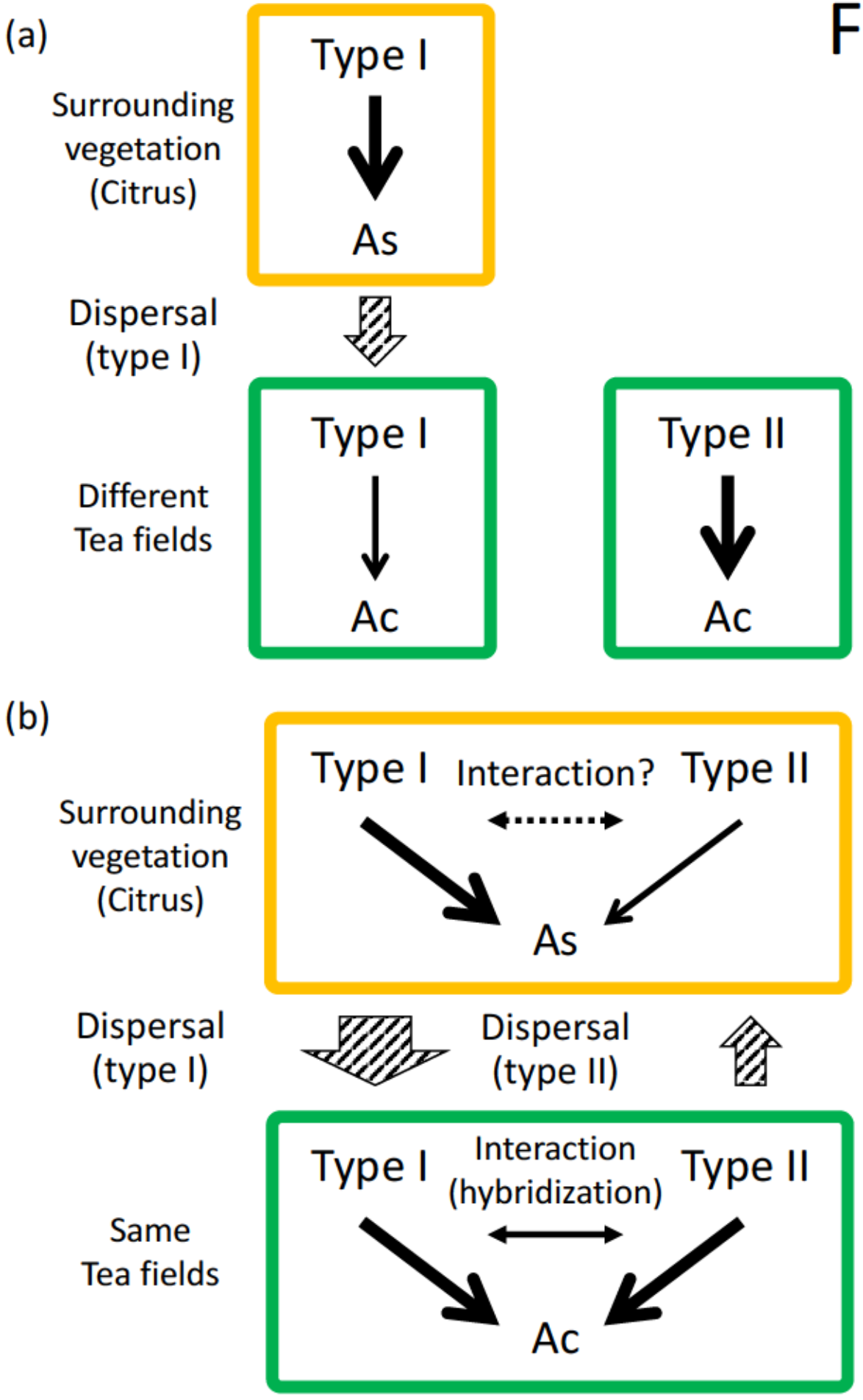

Table 1. *Encarsia smithi* used for DNA sequencing of the nuclear rDNA ITS region

Phylogroup	Code[a]	City	Prefecture	Year	Host	Host plant	*n*
I	SHZ2	Okitsu	Shizuoka	2010	*Aleurocanthus spiniferus*	*Citrus unshiu*	3
I	EHI	Seiyo	Ehime	2010	*A. spiniferus*	*Ci. unshiu*	3
I	OKI	Gesashi	Okinawa	2011	*A. spiniferus*	*Caesalpinia crista*	3
II	GIF	Ogaki	Gifu	2010	*Aleurocanthus camelliae*	*Camellia sinensis*	4
II	MIE	Kameyama	Mie	2009	*A. camelliae*	*Ca. sinensis*	4
II	NAR	Nara	Nara	2010	*A. camelliae*	*Ca. sinensis*	4

[a] Uesugi et al. (2016a)

Table 2. Specific primer pairs and PCR product size in *Encarsia smithi*

Phylogroup	Name	Sequence (5'-3')	Size (bp)
I	ITS1-EsI-F	CACGCAACGTTTTAAACTTTATAC	993
	ITS2-EsI-R	CAAGTCGACCGCGATTAGTC	
II	ITS1-EsII-F	GAAACTTGATACAGAAATTCG	890
	ITS2-EsII-R	CTTTAAAATTTCTCAGAAAGAGG	

Table 3

Table 3. Sample locations and numbers of *Encarsia smithi* analyzed (host - host plant: *Aleurocanthus camelliae* - tea) in this study

No.[a]	City or Town	Jul.-Aug, 2013		Apr.-May, 2014		Aug., 2014		Jul., 2015	
		F[b]	M[c]	F	M	F	M	F	M
1	Numazu_1	16						5	3
2	Numazu_2	15						5	3
3	Numazu_3	11							
4	Numazu_4	5	3						
5	Fujinomiya_1	14						8	
6	Fujinomiya_2	2	5					2	1
7	Fujinomiya_3	15						2	11
8	Fujinomiya_4	10						5	1
9	Shizuoka_1	1	1					6	1
10	Shizuoka_2	6	2					8	
11	Shizuoka_3	16						3	5
12	Shizuoka_4	15						8	
13	Fujieda	22						8	
14	Shimada_1	11						8	
15	Shimada_2	23		29		6		8	
16	Makinohara	15		8					
17	Kawanehoncho	16				8		8	
18	Kikugawa			8					
19	Kakegawa	2	4						
20	Iwata	3	9						
21	Hamamatsu_1	15	1					2	6
22	Hamamatsu_2		2						8
23	Hamamatsu_3	12	1						4
24	Hamamatsu_4	10	5	7				8	
15	Shimada_2						1[d]		

[a] Numbers preceding name of city or town correspond to those in Figure 3.
[b] F: Female.
[c] M: Male.
[d] Obtained from the host *Aleurocanthus spiniferus* infesting a citrus leaf.