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Taiwanese *Trichogramma* of Asian Corn Borer: Morphology, ITS-2 rDNA Characterization, and Natural *Wolbachia* Infection

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Abstract

Egg parasitoids of the genus *Trichogramma* are natural enemies of many lepidopteran borers in agricultural areas around the world. It is important to identify the correct species and ideally focus on endemic *Trichogramma* for pest control in particular crops. In this study, *Trichogramma* wasps were collected from parasitized eggs of Asian corn borer in Southwestern Taiwan. Three *Trichogramma* species, *Trichogramma ostriniae* Pang and Chen, *Trichogramma chilonis* Ishii, and *T.* sp. *y*, were identified based on morphology and the nucleotide sequence of the internal transcribed spacer 2 (ITS-2) region of rDNA. Although *T. ostriniae* and *T.* sp. *y* appear to be morphologically similar, ITS-2 identity between these two taxa is only 89%. Surprisingly, a commercially released *Trichogramma* colony thought to be *T. chilonis* possessed 99% identity (ITS-2) with the field *T.* sp. *y* individuals. This suggests past contamination leading to subsitution of the laboratory-reared *T. chilonis* colony by *T.* sp. *y*. Natural populations of all three *Trichogramma* species were found to be infected by a single *Wolbachia* strain which was identified using a *wsp* gene sequence.

Key words: biological control, egg parasitoid, endosymbiont, ITS-2 rDNA, *Trichogramma*

Trichogramma (Hymenoptera: Trichogrammatidae) are polyphagous endoparasitoids of lepidopteran eggs commonly used for biological control of economic pests around the world (Hoffmann et al. 2001, Kuhar et al. 2004, Zhang et al. 2010, Wang et al. 2014). Because they can be easily reared in the laboratory on hosts other than the field hosts and have a broad host range of target pests, they play an important role in pest control (Mills 2010).

Crop pests targeted by *Trichogramma* include those of sugarcane and corn in southeastern Asia. Sugarcane and corn crops in Taiwan share several lepidopteran pests including Asian corn borer *Ostrinia furnacalis* (Guenée), sugarcane gray borer *Argyroploce schistaceana* Meyrick, and sugarcane stalk borer *Proceras venosatus* Walker. Around 1984, when surplus production of rice in Taiwan led to a change in agricultural policy to replacement of rice with corn and sorghum, control measures for these pests became more important. Mass rearing and release of *Trichogramma chilonis* Ishii had been conducted in the 1950s for *A. schistaceana* and *P. venosatus* control. Following implementation of the corn transfer policy, inundative releases of *T. chilonis* and *Trichogramma ostriniae* Pang and Chen for controlling corn and sugarcane borer were conducted (Cheng 1997): *T. ostriniae* was mass reared and released in those fields for biocontrol in 1980s (Cheng 1997) (Fig. 1). In addition, a strain designated *T.* sp. *y* reported by Kim and Huh (GenBank GQ228084.1) was maintained in the laboratory of Taiwan Sugar Corporation and released regularly in 1950s (Wu et al. 2015, GenBank KU199688). Thus, several *Trichogramma* species are known to have been reared and released. However, because of this complex agricultural history of releases in Southwestern Taiwan combined with the challenges of identifying *Trichogramma* morphologically, there is uncertainty regarding identification of species present and the composition of species complexes contributing to pest control.

Although eight species of *Trichogramma* have been recorded in Taiwan (Chan and Chou 2000), two species, *T. ostriniae* and *T. chilonis*, are the most frequently recorded parasitoids of the Asian crop borers of greatest concern, with natural parasitism of *A. schistaceana* in sugarcane as high as 71% (Cheng 1997, Chang et al. 2001). *Trichogramma* species are notoriously difficult to identify because of their small size (<1 mm) and low interspecific morphological diversity (Thomson et al. 2003): the most commonly used and most reliable morphological characteristics are features of the male genitalia (Chan and Chou 2000). Life history traits such as development time, fecundity, longevity, and host acceptance can also help in distinguishing between species (Smith and Hubbes 1986). More recently, the internal transcribed spacer regions (ITS-1 and ITS-2) of the ribosomal gene have been found to be effective in distinguishing *Trichogramma* species found around the globe (Chang

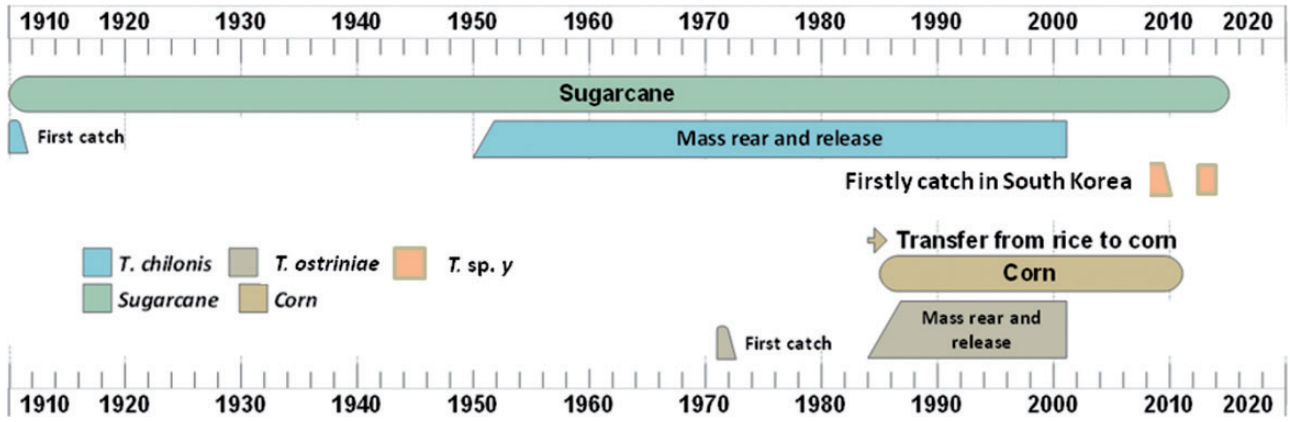

Fig. 1. Agricultural land use and *Trichogramma* parasitoids release history for Southwestern Taiwan. Information modified from Cheng (1997).

et al. 2001, Thomson et al. 2003). The development of better approaches for identifying *Trichogramma* species during the last decades provides as opportunity for revisiting *Trichogramma* used for pest control in Taiwan.

Polymerase chain reaction (PCR) testing with primers for the *Wolbachia wsp* gene revealed both *T. ostriniae* and *T. chilonis* were infected with two groups of *Wolbachia* (Kue and Pip) (α-proteobacteria), maternally inherited intracellular bacteria (Song et al. 2010, Wu et al. 2015). Because of their dependence on host reproduction, natural selection is expected to favor symbionts that are most benevolent to the host—those that least harm host growth rate and other aspects of host fitness (Bull et al. 1991). Accordingly, several thelytoky-inducing *Wolbachia* have been shown to confer benefits on the fitness of their hosts under some conditions; for example, thelytokous strains of *Trichogramma pretiosum* and *Trichogramma cordubensis* lower mortality and increase longevity when compared to sexually reproducing strains (Pintureau and Bolland 2001). Because *Wolbachia* infection can lead to large impacts on wasp fitness, knowledge of the infection status of populations may play an important role in selection of wasps for biological control programs (Hoffmann and Turelli 1990, Stouthamer et al. 1994, Poorjavad et al. 2012). In previous work, we have shown *Wolbachia*-infected *T. ostriniae* from Taiwan tend to remain active longer than uninfected colonies under high temperature conditions (30–50°C) (Wu et al. 2015). Tolerance to higher temperature may be an advantage in Taiwan especially under conditions of climate change.

The aim of this study is to identify *Trichogramma* species emerging from parasitized Asian corn borer eggs collected from southwestern Taiwan using morphological features and molecular methods and to investigate the presence of *Wolbachia* in these species. Detected *Wolbachia* strains were characterized and placed within a broader *Wolbachia* phylogeny based on the *Wolbachia* surface protein (*wsp*) (Braig et al. 1998).

Materials and Methods

Trichogramma Sampling and Collection

Trichogramma were isolated from 1,031 naturally occurring egg masses of *O. furnacalis* found on sweet corn from six sites throughout the Southwestern counties (Changhua, Yunlin, Chiayi, and Tainan) of Taiwan in June of 2014. One of the sites (site A) was sampled on three other occasions during April and June 2013 and December 2014 for examining effects of sampling time on *Trichogramma* species composition and *Wolbachia* infection status (Fig. 2). This site comprised an organic field where *Trichogramma* from a colony managed by Tainan District Agricultural Research and Extention Station had been regularly released.

Species Identification

About 30 wasps emerging from parasitized eggs were collected for morphological identification, and remaining wasps were kept for molecular identification. For morphological identification, wasps were soaked in acetic acid for 2 d and slides were made of genitalia from five of the males: these were examined microscopically (Sorokina and Atamirzaeva 1993). Males were present in all samples.

For molecular identification of wasps with ITS-2 sequence analysis and for characterizing *Wolbachia* infections, genomic DNA was isolated from individual wasps using an ALS Tissue Genomic DNA Extraction Kit (Kaohsiung, Taiwan) and stored at −20°C until use. Primers used for PCR amplification of the ITS-2 rDNA and *wsp* (81F/691R) genes (Braig et al. 1998, Chang et al. 2001, Wu et al. 2015) are given in Supplementary Table S1. PCR was initiated at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 40°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. The PCR products were obtained by electrophoresis in 1.5% agarose gels and sequenced. Sequences were aligned using MEGA 6 software (Tamura et al. 2013).

Phylogenetic and Statistical Analysis

Sequences of the *wsp* gene were identified through a Blast search via the National Center for Biotechnology Information website to determine whether the sequences were *wsp* gene of *Wolbachia*. The *wsp* sequences obtained in this study and 21 reference *wsp* sequences retrieved from GenBank were used to construct a rooted phylogenetic tree (Zhou et al. 1998, Song et al. 2009) with Bayesian inference of phylogeny using MEGA 6. The tree was constructed through maximum-likelihood and neighbor-joining models. Bootstrapping was performed with the heuristic option for 500 replications in the two models.

Statistical analyses were performed in R (version 3.1.1, R Core Team 2014). We compared wasp species composition and *Wolbachia* infection rates in *Trichogramma* species using G-tests with sequential Bonferroni correction.

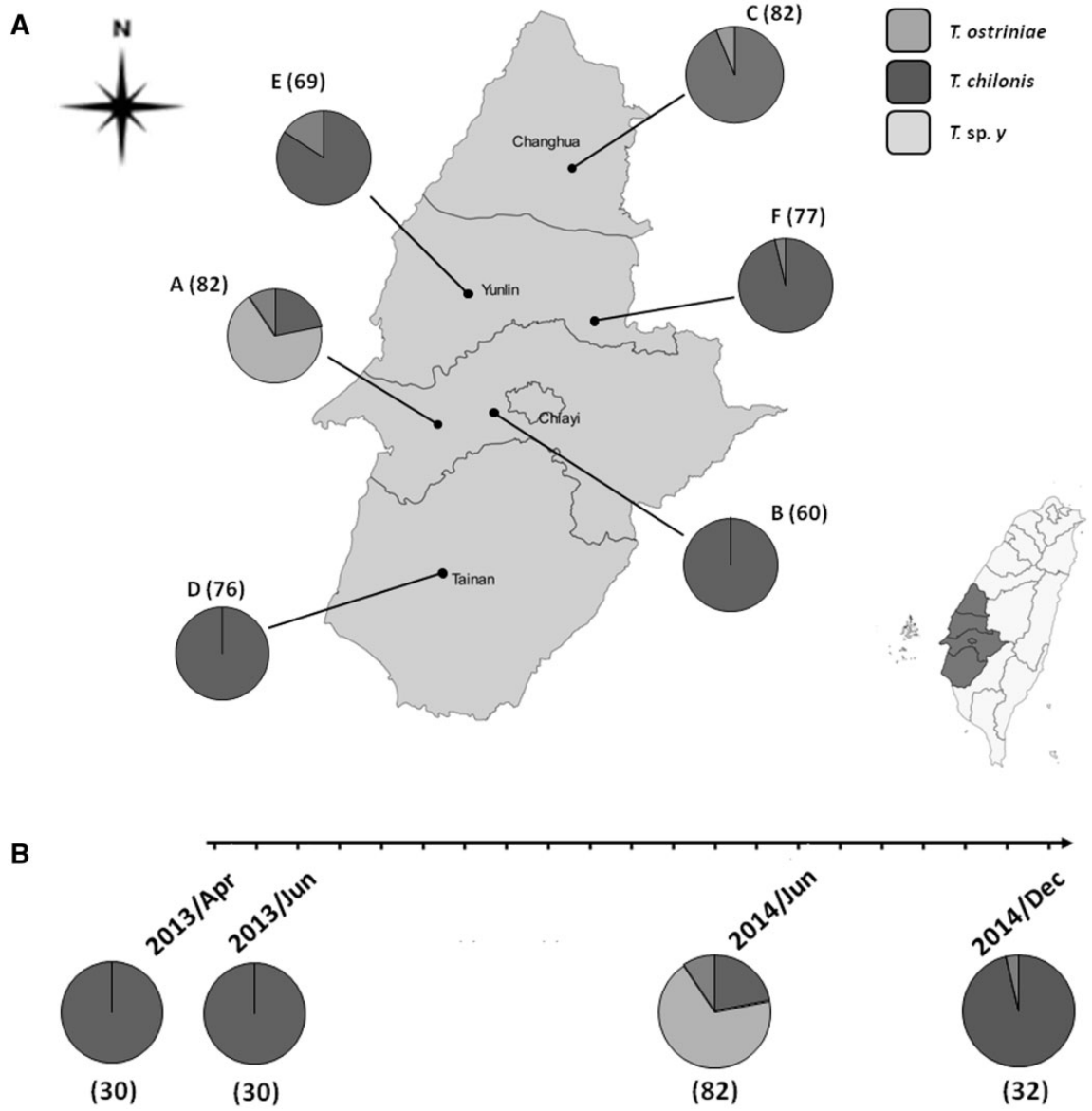

Fig. 2. (A) Species composition of *Trichogramma* species emerging from field collected naturally parasitized egg masses of *O. furnacalis* collected from sweet corn throughout the Southwestern counties (Changhua, Yunlin, Chiayi, and Tainan) of Taiwan (sites A–F) in June (2014). (B) Species composition at site A across other three sampling times. Species were determined by ITS-2 sequence analysis as given in the pie diagrams. Sample sizes (brackets) range from 30 to 82.

Results

Morphological and Molecular Identification of Trichogramma Species

On the basis of male genitalia features, wasps could be divided into two species: *T. ostriniae* and *T. chilonis*. Species identification was confirmed by ITS-2 sequence analysis. The third species, *T.* sp. *y*, possessed similar genitalia traits to *T. ostriniae:* moderately constricted base of dorsal lamina, with the absence of narrowly rounded lateral lobes seen in *T. chilonis* (Fig. 3). However, *T.* sp. *y* could not be assigned confidently to *T. chilonis* because there were minor differences in their ITS-2 sequence (see Wu et al. 2015, GenBank KU199688).

Following *Trichogramma* species identification based on ITS-2 sequencing and morphological comparisons, a total of 341 *Trichogramma* individuals were identified morphologically and molecularly as *T. ostriniae*, 39 individuals as *T. chilonis*, and 66 individuals as *T.* sp. *y*, based on wasps reared from 1,031 parasitized egg masses of *O. furnacalis*. *T. ostriniae* individuals were identified from all six locations and all four sampling times, and *T. chilonis* individuals were identified from four northern locations (A, C, E, and F) and two of four sampling times. *T.* sp. *y* occurred at a single site, site A. The species composition was compared across the six sampling sites and shown to differ ($G_{10} = 90.46$; $P < 0.001$) (Fig. 2A). *T. ostriniae* was most abundant at all sites apart from site A where *T.* sp. *y* predominated; *T. chilonis* was found at four of six sampling sites.

A difference in species composition was also detected among the four sampling times ($G_6 = 91.43$; $P < 0.001$). *T. ostriniae* was the most abundant species in three of the four samples, while *T. chilonis* was captured at one of the four sampling times, as was *T.* sp. *y* which was the dominant species in the June sample (Fig. 2B). The species composition remained consistent among rest of three

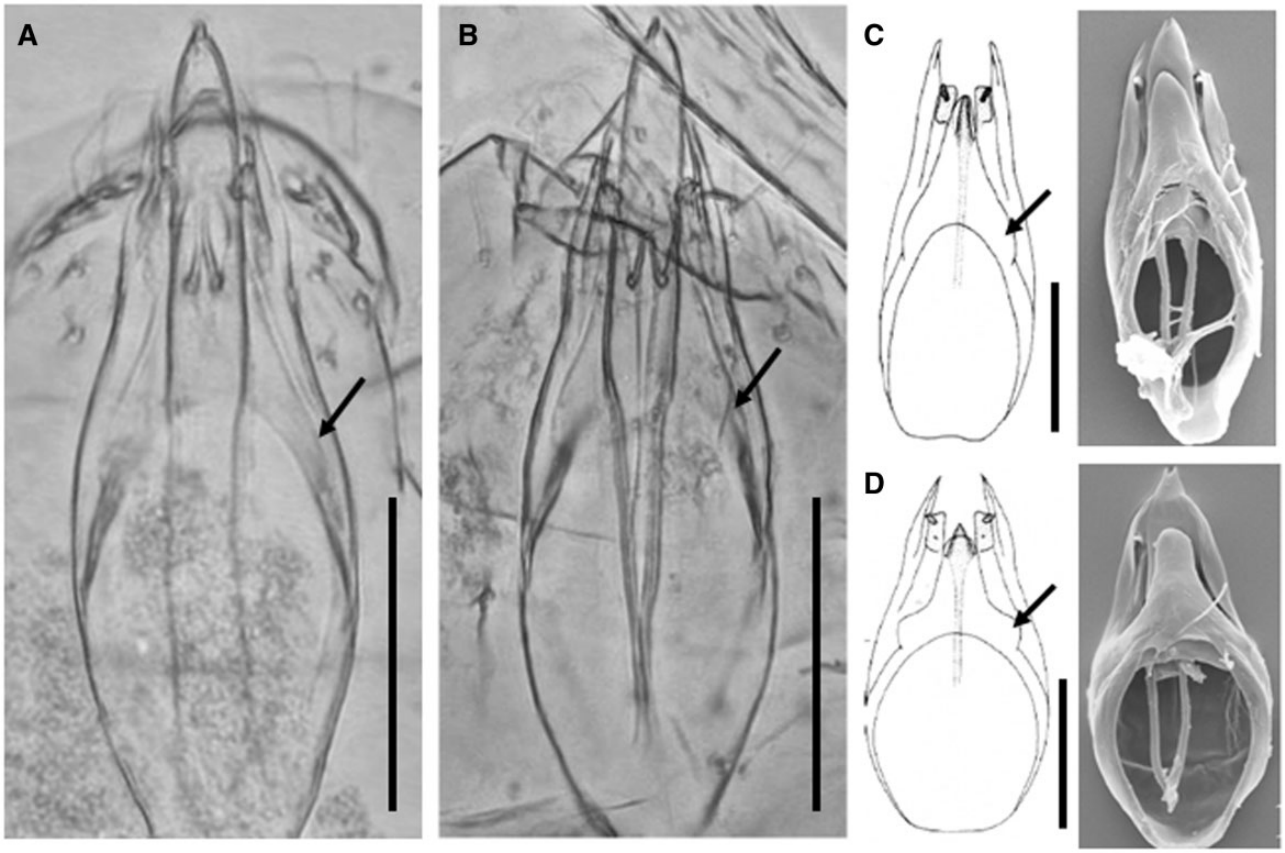

Fig. 3. Dorsal view of genitalia of (A) *T. ostriniae*, (B) *T.* sp. *y*, (C) *T. ostriniae* modified from (Chan and Chou 2000, Honda et al. 2006), and (D) *T. chilonis* modified from Chan and Chou (2000) and Honda et al. (2006). Note similarity of moderately constricted shoulder of dorsal lamina (black arrow). DLA, dorsal lamina; bar = 50 μm.

sampling time after we excluded the *T.* sp. *y* dominated sample ($G_4 = 3.22$; $P = 0.521$).

Phylogenetic Analysis of Wolbachia Infection

A PCR assay was carried out to detect *Wolbachia* in the three species of *Trichogramma*. In total, 238 individual wasps from nine populations were tested; 146 from the six site collections and a further 92 from the time series collection from site A. The infection was detected in 56 individuals from the six sites and 40 from the time series. Samples of all three *Trichogramma* species were found to be infected, but the same *Wolbachia* sequence was identified from each species. Similarity with *wsp* gene sequences retrieved from GenBank was greater than 95%. Phylogenetic analyses using both models yielded a similar topology. The *Wolbachia* sequence belongs to group *Pip* in supergroup B, and it is identical with the sequence provided by Song et al. (2009) for *T. ostriniae* from mainland China (Fig. 4).

Wolbachia Infection Rate Over Different Sites and Time

The *Wolbachia* infection rates for samples from the species varied: 35.6% (95% binomial confidence intervals: 30.0%, 42.6%, $n = 222$) in *T. ostriniae,* 4.6% (0.6%, 26.2%, $n = 22$) in *T.* sp. *y*, and 15.4% (3.9%, 45.1%, $n = 13$) in *T. chilonis*. The *Wolbachia* detected in *T. chilonis* and *T.* sp. *y* came from two individuals of each of these species collected from site A. For the infection in *T. ostriniae*, no infection was detected at site F, and this contributed to significant heterogeneity in infection rate across sampling sites ($G_5 = 41.91$; $P < 0.001$) (Fig. 5). Infection rate varied between 42.9 and 68.8% at the other five sites, and *Wolbachia* infections across these sites did not differ significantly ($G_4 = 2.24$; $P = 0.691$); however, the emergence rate of *T. ostriniae* for some sites (site A and site B) was low, resulting in small sample sizes ($n = 7$ and 10).

Among the four samples from site A, the *Wolbachia* infection rate was not significantly different among the dates ($G_3 = 0.80$; $P = 0.850$) (Fig. 6).

Discussion

Three *Trichogramma* species (*T. ostriniae*, *T. chilonis,* and *T.* sp. *y*) were identified in this field survey, in which similar rates of parasitism were observed among sampling sites and times. Our results, consistent with Cheng (1997) and Wu et al. (2015), indicate that *T. ostriniae* is the dominant wasp parasitoid of Asian corn borer, with *T. chilonis* co-occurring at several sites. According to Cheng (1997), *T. ostriniae* has been collected from eggs of several economic crop pests incuding Asian corn borer, and also *A. schistaceana, P. venosatus,* and striped stem borer *Chilo suppressalis* (Walker), from maize, rice, and sugarcane fields in Southwestern Taiwan. *T. ostriniae* has been extensively used in inundative release programmes against the sugarcane stalk borer *P. venosatus*, the sugarcane gray borer *A. schistaceana,* and the Asian corn borer *O. furnacalis* (Cheng 1997). In Taiwan, *T. ostriniae* are known mainly from corn/maize fields (Cheng 1997, Wu et al. 2015), while *T. chilonis* are known mainly from rice fields (Cheng 1997).

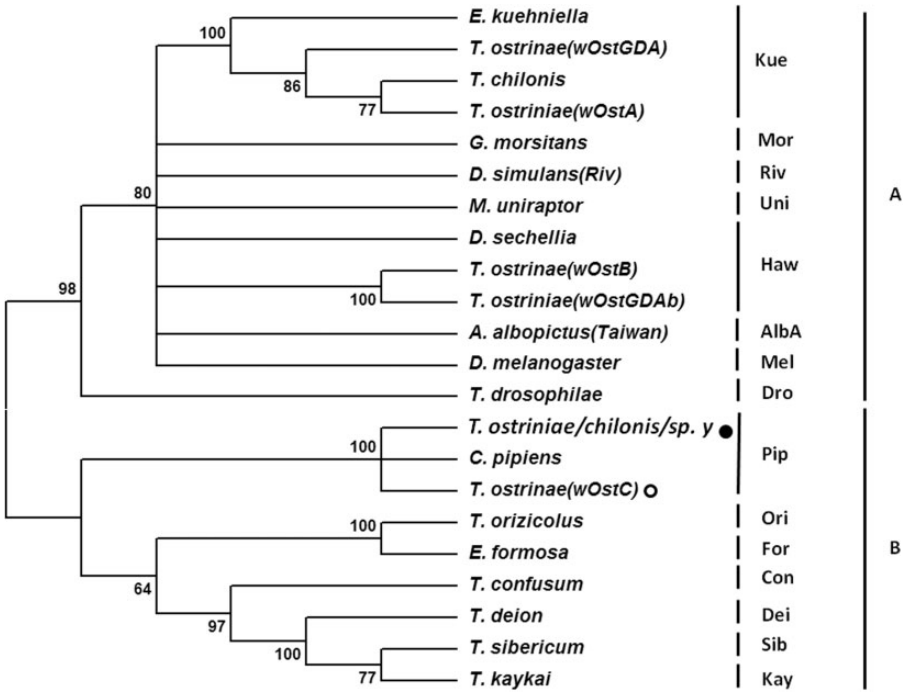

Fig. 4. Phylogenetic tree of *Wolbachia* based on *wsp* gene constructed with the maximum-likelihood method. The sequence obtained in this study is indicated by a filled circle and was detected from all three *Trichogramma* species screened. The sequence obtained from Song et al. (2009) is indicated by an open circle.

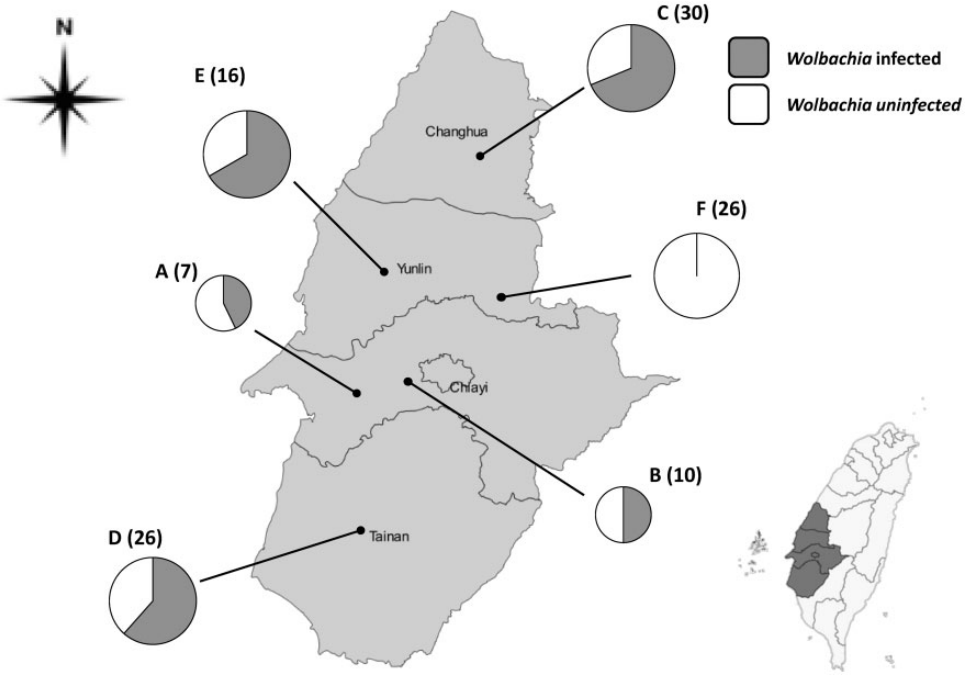

Fig. 5. *Wolbachia* infection rate of *T. ostriniae* from field-collected parasitized egg masses of *O. furnacalis* collected from sweet corn throughout the Southwestern counties (Changhua, Yunlin, Chiayi, and Tainan) of Taiwan (sites A–F) in June 2014 given in the pie diagrams. Sample sizes (brackets) range from 7 to 30.

This study indicated that the ITS-2 region provides an efficient approach for separating closely related *Trichogramma* parasitoids of Asian corn borer in Taiwan, consistent with the effectiveness of this marker in distinguishing other *Trichogramma* species (e.g., Thomson et al. 2003). *T. ostriniae*, *T. chilonis*, and *T.* sp. *y* were difficult to distinguish morphologically but were all easily separated on the basis of their sequence differences. Moreover, ITS-2 sequence allowed identification of female *Trichogramma* spp. which is not possible using external morphological characters.

Trichogramma sp. *y* was only collected from one site (site A) in our study. The species has previously been reported from both South Korea and Taiwan (see Wu et al. 2015). Suprisingly, the laboratory-

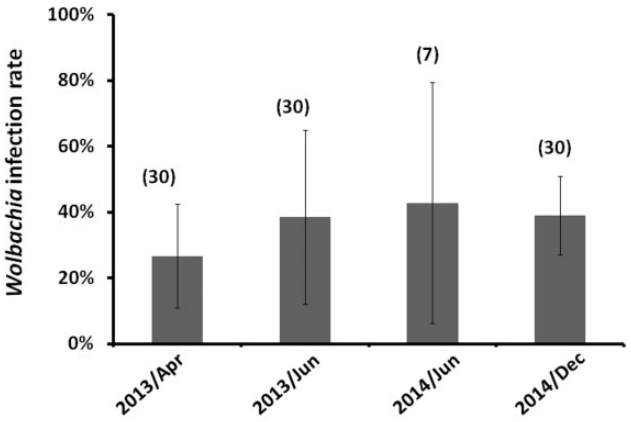

Fig. 6. *Wolbachia* infection rate (% ± CI) remained consistent within *T. ostriniae* at site A across sampling time ($n = 97$). Sample sizes (brackets) range from 7 to 30. Error bars indicate 95% binomial confidence intervals (CIs, binomial test).

reared *Trichogramma* colony from the Taiwan Sugar Corporation (government-owned sugarcane growing organization) possessed 99% identity (ITS-2) with our field individuals from site A (Wu et al. 2015). The Taiwan Sugar Corporation is reported as rearing and releasing *T. chilonis* to control the sugarcane pests (*A. schistaceana* and *P. venosatus*) since 1950 and has achieved parasitism rates of up to 58.9% in the field (Cheng 1997). However, this work suggests that there may have been contamination at some point in time, leading to subsitution of the laboratory-reared *T. chilonis* colony by *T.* sp. *y*. Information provided by the Taiwan Sugar Corporation indicates that the laboratory *T. chilonis* colony has been refreshed from field collected individuals every 2–3 yr since 1972 (Cheng 1997) which may have resulted in contamination. Our findings reinforce the notion that it is important to monitor species destined for release in order to detect contamination events, particularly when local endemic *Trichogramma* are being reared for pest control in particular crops (Wang et al. 2014). In some cases, release of the wrong species in an area where another closely related species is present might even lead to a suppression of both native and introduced species in biological control programs (Stouthamer et al. 2000), although we are unaware of whether inundative releases of *T.* sp. *y* have had negative consequences.

Based on the current field survey, *T. ostriniae* and *T. chilonis* both appear to be active in corn in Southwestern Taiwan on egg masses of *O. furnacalis*. *T. chilonis* has been reported from eggs or egg masses of diverse species including *Orgyia postica* (Walker), *Lampides boeticus* (L.), *Ochyrotica yanoi* (Arenberger), *Protoparca convolvuli* (L.), *Plutella xylostella* (L.), *Trichoplusia ni* (Hubner), *A. schistaceana*, *P. venosatus*, *C. suppressalis*, and *Cnaphalocrocis* (Chen and Chiu 1986, Cheng 1997, Chan and Chou 2000). However, our data suggest that *T. chilonis* may not occur in southern sites, unlike *T. ostriniae* (Fig. 2A). This is consistent with museum records which indicate that *T. ostriniae* tend to be distributed further south than *T. chilonis* in Taiwan (Chan and Chou 2000). In contrast to *T. ostriniae*, *T. chilonis* predominates in rice fields (Chen and Chiu 1986). Historically, rice and corn fields have been cultivated close to each other in Southwestern Taiwan, and this may have affected *Trichogramma* species composition (Cheng 1997). On the other hand, the presence of *T.* sp. *y* only at site A (Puzi) may reflect the fact that this site is a long-term organic study field that routinely releases *Trichogramma* species; this site is managed by Tainan District Agricultural Research and Extention Station, which obtains wasps for inundative release from the Taiwan Sugar Corporation (Cheng 1997), and *T.* sp. *y* at this site may have originated from the laboratory-reared *T.* sp. *y* colony. However, *T.* sp. *y* was not captured at other sample sites, even though inudative releases (most likely involving *T.* sp. *y* unless there has been a recent contamination) would have taken place throughout our sampling area in Southwestern Taiwan. Perhaps, competition with other species in corn fields limits the dispersal and establishment of *T.* sp. *y* in this crop. The relationship between *T. ostriniae* and *T.* sp. *y* is still ambiguous and requires more work.

Based on the PCR surveys of *wsp* gene, this study indicated w*Pip Wolbachia* infections present in all *Trichogramma* species, *T. ostriniae*, *T. chilonis*, and *T.* sp. *y*, and a consistent infection rate of around 35%. *Wolbachia* prevalence differed from the Song et al. (2009) where all of individuals (101) from Guangdong, China, were triple infected by *wKue*, *wHaw*, and w*Pip* group *Wolbachia* in *T. ostriniae*, although no triple infected wasps were found in other provinces of China. A high level of infection by *wKue*, *wEva*, and w*Pip* group *Wolbachia* was also found in *T. chilonis* samples from South and North China (Guangdong and Heibei) (Song et al. 2010).

In conclusion, we found that Taiwan's most abundant *Trichogramma* in corn, *T. ostriniae* and *T. chilonis*, can be successfully separated by specific molecular primers based on ITS-2 sequences (Chang et al. 2001). Although *T. ostriniae* and *T.* sp. *y* appear to be morphologically similar and with a similar life history (Wu et al. 2015), the ITS-2 identify between these two species is only 89%. Given the absence of *T.* sp. *y* from most field sites, a different species may need to be used for mass rearing. *Wolbachia* bacteria have infected some populations of *T. ostriniae*. *T. chilonis*, and *T.* sp. *y* collected from Southwestern Taiwan. As the *Wolbachia* infection rate is relatively consistent across *T. ostriniae* populations, this situation provides an opportunity to identify factors maintaining stable polymorphic *Wolbachia* infections in populations.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

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