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ORIGINAL RESEARCH PAPER

Existence of two strains of *Habrobracon hebetor* (Hymenoptera: Braconidae): a complex in Thailand and Japan

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Abstract
Habrobracon hebetor Say (Hymenoptera: Braconidae) is a cosmopolitan gregarious ectoparasitoid that attacks larvae of several species of Lepidoptera. Although there are two genetically different strains within *H. hebetor*, distribution of the strains has been poorly understood. In 2010, in Thailand, where *H. hebetor* has been known as a parasitoid of stored grain pests, it was found that *H. hebetor* attacked *Opisina arenosella* Walker (Lepidoptera: Oecophoridae), which is an invasive pest of coconut palm. For correct identification of this *H. hebetor*, we conducted DNA analysis and cross tests using populations collected from *O. arenosella* and stored grain pests in Thailand and populations in Japan known as *H. hebetor*. We obtained 413 bp of mitochondrial cytochrome oxidase I (*COI*) sequences and 414 bp of 16S rRNA gene sequences, and both indicated that there are two distinct clades within *H. hebetor*: one contains insects from Thailand, Spain, India, and Barbados; the other contains insects from Japan and the USA. There were no genetic differences or sexual isolation between Thai populations from different hosts. Our results also showed that populations in Thailand were sexually isolated from a *H. hebetor* population in Japan.

Keywords Biologic control · Clade · Coconut · Parasitoids · Strain

Introduction

Accurate identification of species of natural enemies is one of the key points in successful biological control (Van Driesche and Hoddle 2000). A natural enemy species may contain strains that are morphologically indistinguishable. Such strains may have different biological characteristics, including survival, fecundity and host preference, that affect the efficacy of biocontrol (Hoelmer and Kirk 2005; Sarfraz et al. 2005). Identification of species or strains using not only morphologic traits but also genetic traits is thus important for developing an effective control strategy.

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Habrobracon hebetor Say (Hymenoptera: Braconidae) is a cosmopolitan gregarious ectoparasitoid that attacks larvae of several species of Lepidoptera. In Japan and the USA, it has been reported that this parasitoid normally attacks stored grain pests, including *Corcyra cephalonica* Stainton (Lepidoptera: Pyralidae), *Plodia interpunctella* (Hübner) (Pyralidae), *Ephestia cautella* (Walker) (Pyralidae) and *Pyralis farinalis* (Linnaeus) (Pyralidae) (Brower et al. 1996; Tamura 1994; Watanabe 1933). However, a wide range of *H. hebetor* hosts, including noctuid or gelechiid spp., has been reported on outdoor crops in the Sahel, Italy, Israel, Iraq, Azerbaijan and India (Al-Maliky and Al-Izzi 1986; Gahukar et al. 1986; Gerling 1971; Mamedov 1989; Loni et al. 2016; Puttarudriah and Basavanna 1956). Heimpel et al. (1997) reported that there are two genetically different strains of *H. hebetor*: one is distributed in the USA and the other in Barbados. They are sexually isolated and have different biologic traits. Distribution of the two strains other than in the USA and Barbados is unknown.

In Thailand, *H. hebetor* has been known to be a parasitoid species of stored grain insect pests including *C. cephalonica* and *Sitotroga cerealella* (Olivier) (Konishi et al. 2004). In 2010, it was first found in Thailand that *H. hebetor* attacked

the coconut black-headed caterpillar, *Opisina arenosella* Walker (Lepidoptera: Oecophoridae), which is an exotic pest species causing heavy damage in coconut plantations in central and southern Thailand (IPPC 2017). *H. hebetor* was found to attack *O. arenosella* in India (Nasser and Abdurahiman 2001), and since then this parasitoid has been used to control *O. arenosella* in Thailand (IPPC 2017). Although this parasitoid has been identified as *H. hebetor* based on morphological study (K, Chareonsom, personal communication), identification with DNA analysis has not been conducted.

To reveal the genetic difference among populations in Thailand, we conducted DNA analysis with the mitochondrial cytochrome oxidase I (*COI*) gene and 16S rRNA gene of several populations of *H. hebetor* collected from *O. arenosella* and from stored grain pests. We also compared the biological traits of the Thai and Japanese populations, which are genetically different.

Materials and methods

Insects

Adult *H. hebetor* were obtained from six colonies in Thailand and two populations from Japan (Table 1). Colonies in Thailand were initiated from *H. hebetor* collected in different locations in Thailand and were maintained on the fifth instar larvae of *C. cephalonica* before experiments. Insects collected in Tokyo, Japan, were maintained on several hosts including *C. cephalonica* and *P. interpunctella* before experiments.

DNA analysis

To extract the total genomic DNA, the whole body of female and male adults of *H. hebetor* was homogenized in 100 µl 5% Chelex® 100 resin (Bio-Rad Laboratories, Inc., CA, USA), and then 2 µl 20 mg/ml proteinase K was added. The mixture was incubated overnight and then for 3 min at 99.9 °C. To compare with previously reported sequences of *H. hebetor* from different locations, we used two sets of primers that amplify the mitochondrial cytochrome oxidase I (*COI*) gene or 16S rRNA gene. For *COI* forward (LCO1490) 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′ and reverse (HCO2198) 5′- TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ (Folmer et al. 1994) and for the 16S rRNA gene forward (16sWb) 5′- CAC CTG TTT ATC AAA AAC AT -3′ and reverse (16s.Sh) 5′- AGA TTT TAA AAG TCG AAC AG-3′ (Heimpel et al. 1997) were used. The 20-µl PCR reaction mixture contained 10× ExTaq buffer (Takara Bio, Otsu, Japan), 0.2 mM dNTP mixture, 0.5 µM of each primer, 0.5 U TaKaRa ExTaq polymerase and 2.0 µl DNA solution. PCR was conducted with 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 90 s; a final 72 °C for 7 min for *COI* and 54 °C of annealing temperature were used instead for the 16S rRNA gene. PCR products were sent to FASMAC Co., Ltd., Atsugi, Japan, for sequencing.

The obtained *COI* and 16S rRNA gene sequences were aligned by using the CLUSTAL X (Thompson et al. 1997). Phylogenetic trees were constructed for each region by the neighbor-joining method (Saitou and Nei 1987) with MEGA6 software (Tamura et al. 2013) with other *H. hebetor* and sequences of high similarity (< 99%) detected by BLASTn. For outgroups, *Bracon tamabae* Maeto (LC020186.1) and *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) (KJ882548.1) were used for *COI*, and *B. phylacteophagus* (AF003481.1) and *C. flavipes* (KJ882489.1) were used for the 16S rRNA gene. The evolutionary distances were computed by the Kimura two-parameter method, which accounts for differing rates of transition versus transversion mutations (Kimura 1980). Nodal support was evaluated with 1000 bootstrap resamplings (Felsenstein 1985). The sequence data were deposited

Table 1 Source locations of the colonies of *Habrobracon hebetor* used for DNA analysis and crossing

ID	Location	Latitude (N)	Longitude (E)	Host species	Date of collection	Accession no. (n)	
						For *COI*	For 16S
TOA1	Thailand, Prachuapkhirikhan	11.68	99.72	*Opisina arenosella*	14 Dec. 2010	LC132718 (5)	LC132720 (2)
TOA2	Thailand, Prachuapkhirikhan	12.07	99.86	*Opisina arenosella*	19 Dec. 2014	–[a] (6)	–[b] (2)
TCC1	Thailand, Nakhon Pathom	14.03	99.98	*Corcyra cephalonica*	20 June 2007	–[a] (3)	–[b] (1)
TCC2	Thailand, Suratthani	9.72	100.00	*Corcyra cephalonica*	17 Dec. 2014	–[a] (3)	–[b] (3)
TCC3	Thailand, Bangkok	13.84	100.58	*Corcyra cephalonica*	14 Jan. 2015	–[a] (4)	–[b] (2)
TCC4	Thailand, Singburi	14.8	100.29	*Corcyra cephalonica*	6 Feb. 2015	–[a] (4)	–[b] (3)
JPN	Japan, Tokyo	–	–	–	Oct. 2004	LC341290 (6)	LC341289 (2)
JB	Japan, Ibaraki	–	–	–	–	LC132719 (3)	LC132721 (4)

[a]Sequences are identical to LC132718

[b]Sequences are identical to LC132720

in the DDBJ/EMBL/GenBank database with accession nos. LC132718–LC132721, LC341289 and LC341290.

Reproductive isolation among different populations

To explore reproductive isolation among different populations, crossing tests were conducted with three populations of *H. hebetor*: the two Thai populations initiated with parasitoids collected from the hosts *O. arenosella* or *C. cephalonica* (TOA1 or TCC4 in Table 1) and one Japanese population (JPN in Table 1).

Newly emerged wasps were paired 2 h after emergence. They were introduced into a plastic cup (70 mm diameter, 40 mm height) that contained three fifth-instar larvae of *C. cephalonica* and allowed for mating and oviposition for 48 h. The number of eggs deposited on the hosts, survivorship from egg-to-adult emergence and sex ratio of offspring were recorded and analyzed. A total of 20 pairs were investigated for each cross: TOA1 female × TOA1 male, TOA1 female × TCC4 male, TOA1 female × JPN male, TCC4 female × TCC4 male, TCC4 female × TOA1 male, TCC4 female × JPN male, JPN female × JPN male, JPN female × TOA1 male and JPN female × TCC4 male.

Host use and suitability

Parasitism of two different hosts was compared between a Thai (TOA1) and the Japanese population (JPN) (Table 1). One pair of 1-day-old adults was placed into a plastic cup (70 mm diameter, 40 mm height) that contained one host larva of *C. cephalonica* or *P. interpunctella* and a cotton ball soaked in 5% honey. The adult parasitoids were allowed to mate and oviposition for 24 h. For the next 7 days, hosts were replaced with fresh ones daily. All experiments were conducted at 25 ± 2 °C with a 16L:8D photoperiod.

Data analysis

The percentage of adult emergence in cross tests was analyzed by logistic regression. The sex ratio of offspring produced by mated females was also analyzed by logistic regression. The effect of crosses on those ratios was compared with the likelihood ratio Chi-square tests. In the tests of host use and suitability, we used a generalized linear mixed model (GLMM) to analyze the number of eggs laid in 7 days with Poisson error distribution and log-link function and percentages of adult emergence and female ratios with binomial error distribution and logit-link function. Days after adult emergence was used as a random factor. These analyses were conducted with version 3.4.3 of the R software (R Core Team 2017) with the "lme4" package (Bates et al. 2015).

Results

DNA analysis

We obtained 413 bp of *COI* sequences from 25 insects from Thai populations and 9 insects from the Japanese population (LC132718, LC132719, LC341290) and 414 bp of 16S rRNA gene sequences from 13 insects from Thai populations and 6 insects from the Japanese population (LC132720, LC132721, LC341289). All sequences in each *COI* or 16S rRNA gene were identical in specimens collected in Thailand (Table 1).

Both phylogenetic trees created by *COI* and 16S rRNA gene sequences showed that *H. hebetor* collected in Thailand and Japan belonged to different clades with 100% bootstrap supports for *COI* and 99 and 74% bootstrap supports for 16S rRNA sequences (Fig. 1). According to the tree created by the *COI* sequence, the clade that contained *H. hebetor* collected in Thailand contained Braconinae sp. collected in Spain and *H. hebetor* collected in India (Fig. 1). The tree for the 16S rRNA gene showed that *H. hebetor* in Thailand is in the same clade as *H. hebetor* in Barbados (Heimpel et al. 1997), while the clade of *H. hebetor* in Japan contains *H. hebetor* in the USA (Heimpel et al. 1997) (Fig. 1). The pairwise distance within a clade was 0% for Thai populations and 0.24% for Japanese populations for *COI*. For 16S, it was also 0% for the Thai populations and 0.24% for the Japanese populations, while the pairwise distances between the Thai and Japanese populations were 7.02% (Thailand-JPN) and 6.78% (Thailand-JB) for COI and 2.42% (Thailand-JPN) and 2.17% (Thailand-JB) for 16S.

Reproductive isolation among different populations

No mating was observed in the crosses of populations from different countries, and they produced only male offspring, while crosses of the same country produced both female and male offspring (Table 2). The percentage of adult emergence was not affected by crosses in each parent female ($\chi^2 = 3.936$; df = 2; $p = 0.140$ for the TOA1 parent female, $\chi^2 = 5.597$; df = 2; $p = 0.061$ for the TCC4 parent female, and $\chi^2 = 4.63$; df = 2; $p = 0.099$ for the JPN parent female). In crosses of Thai populations, the female ratio did not differ between crosses of TOA1 parent females ($\chi^2 = 0.3538$; df = 1; $p = 0.552$), while it was higher in the inter-population crosses than in intra-population crosses for TCC4 parent females ($\chi^2 = 59.84$; df = 1; $p < 0.001$) (Table 2).

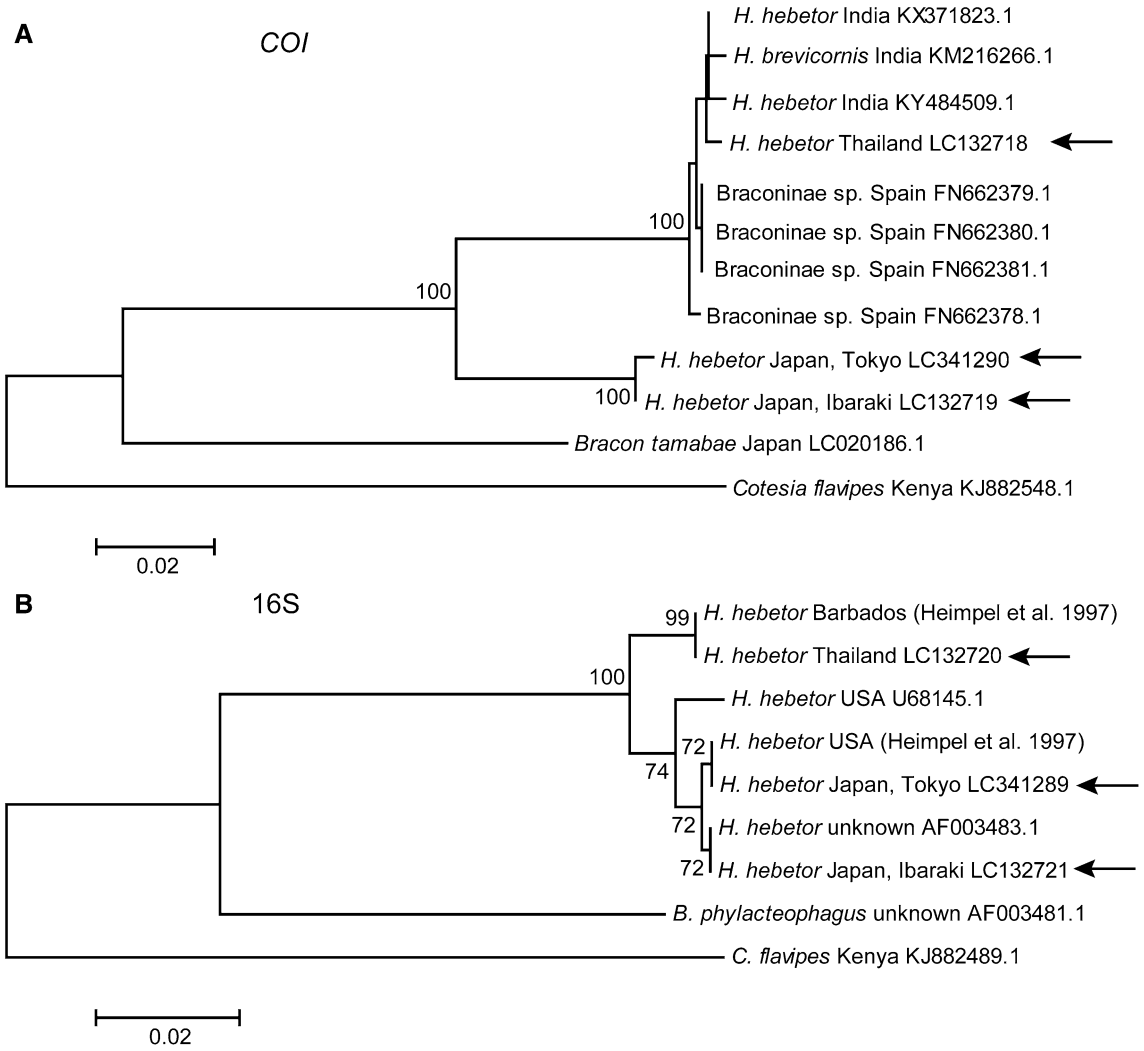

Fig. 1 Phylogenetic position of *Habrobracon hebetor* collected from Thailand and Japan. **a** Tree created using the 413 bp of *COI* sequences. **b** Tree created using the 414 bp of 16S rRNA gene sequences. Arrows indicate new sequences of the current study. The evolutionary history was inferred using the neighbor-joining method with the Kimura two-parameter method. Bootstrap values (>50%) calculated on the basis of 1000 replications are indicated near the branches

Host use and suitability

All *B. hebetor* females from two populations paralyzed and parasitized on both *C. cephalonica* and *P. interpunctella* larvae. However, in JPN, some females did not paralyze and parasitize hosts in a few days after emergence (Fig. 2). The proportion of females attacking hosts in JPN was significantly affected by days after emergence on both host larvae ($\chi^2 = 12.12$; df = 1; $p < 0.001$ for *C. cephalonica*, $\chi^2 = 12.51$; df = 1; $p < 0.001$ for *P. interpunctella*). On the other hand, in TOA1, all the females paralyzed and parasitized hosts during the experimental period, except TOA1 on *P. interpunctella* for the first 2 days (Fig. 2). The number of eggs laid on a host increased with increasing days after emergence until 4 days in TOA1 and JPN on both hosts (Fig. 3). In both TOA1 and JPN, the number of eggs produced in 7 days was larger on *C. cepnalonica* than on *P. interpunctella* ($\chi^2 = 235.03$; df = 1; $p < 0.001$ for TOA1, $\chi^2 = 119.78$; df = 1; $p < 0.001$ for JPN) (Table 3). The percentage of adult emergence and female ratio were higher on *C. cephalonica* than on *P. interpunctella* in TOA1 ($\chi^2 = 54.37$; df = 1; $p < 0.001$ for adult emergence, $\chi^2 = 17.77$; df = 1; $p < 0.001$ for female ratio), while they did not differ in JPN ($\chi^2 = 0.391$; df = 1; $p = 0.532$ for adult emergence, $\chi^2 = 3.261$; df = 1; $p = 0.071$ for female ratio).

In each host species, TOA1 laid more eggs than JPN on *C. cephalonica*, while it did not differ on *P. interpunctella* ($\chi^2 = 26.99$; df = 1; $p < 0.001$ for *C. cephalonica*, $\chi^2 = 0.597$; df = 1; $p = 0.440$ for *P. interpunctella*). Percentages of adult emergence were higher in TOA1 than in JPN on each host

Table 2 Results of the intra- and inter-population crosses of *Habrobracon hebetor*

Cross[a]		No. of pairs	Percentage of females producing female offspring	Survival rates and sex ratios of F_1 offspring			
Female	Male			*n*	Adult emergence[b] (%)	*n*	Sex ratio[b] (% female)
	TOA1	20	65	403	69.4 ± 5.4a	13	57.3 ± 7.4a
TOA1	TCC4	20	75	417	73.7 ± 5.0a	15	63.8 ± 6.9a
	JPN	20	0	415	72.7 ± 5.3a	0	–
	TCC4	20	75	427	63.2 ± 4.6a	15	38.3 ± 5.0a
TCC4	TOA1	20	60	430	64.4 ± 4.0a	12	74.4 ± 4.9b
	JPN	20	0	420	62.0 ± 5.3a	0	–
	JPN	20	90	269	52.7 ± 3.6a	18	67.6 ± 4.6
JPN	TOA1	20	0	295	56.5 ± 6.7a	0	–
	TCC4	20	0	252	52.9 ± 5.6a	0	–

[a]See Table 1 for details of populations (TOA1 and TCC4 from Thailand and JPN from Japan)

[b]Values are mean ± SE. Values in a column followed by the same letters do not differ significantly among crosses within each parent female (likelihood ratio Chi-square test, $p > 0.05$)

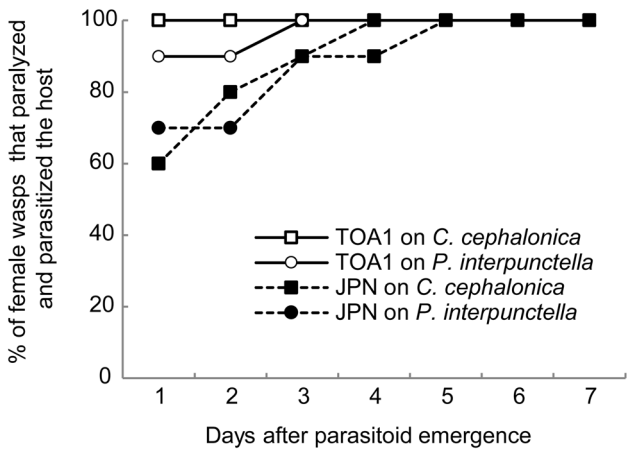

Fig. 2 Percentage of female *Habrobracon hebetor* that paralyzed and parasitized two host species, *Corcyra cephalonica* or *Plodia interpunctella*, at different times after emergence. TOA1 is a population in Thailand, and JPN is a population in Japan (Table 1)

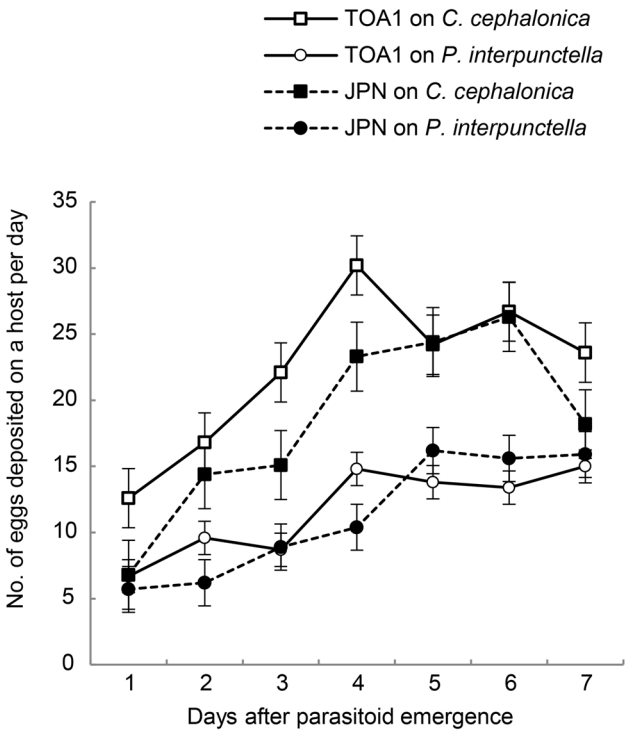

Fig. 3 Number of eggs laid by a female *Habrobracon hebetor* on two host species, *Corcyra cephalonica* and *Plodia interpunctella*, at different times after emergence. Bars are SE. TOA1 is a population in Thailand, and JPN is a population in Japan (Table 1)

species ($\chi^2 = 303.86$; df = 1; $p < 0.001$ for *C. cephalonica*, $\chi^2 = 47.43$; df = 1; $p < 0.001$ for *P. interpunctella*). The female ratio was lower in TOA1 than in JPN on each host species ($\chi^2 = 21.25$; df = 1; $p < 0.001$ for *C. cephalonica*, $\chi^2 = 21.22$; df = 1; $p < 0.001$ for *P. interpunctella*).

Discussion

DNA analysis and cross tests demonstrated that there were no genetic differences or sexual isolation between Thai populations collected from different locations and different hosts, including the population collected from the novel host *O. arenosella* and those from stored grain pests. In Thai populations, both the *COI* and 16S sequences were identical among all populations (Table 1). Although this may indicate that the genetic diversity of *H. hebetor* in Thailand is extremely small, we cannot exclude the possibility of an effect of long-term laboratory rearing. All the samples used for this study were obtained from laboratory colonies that have been continuously reared for a long period, which may result in a reduction of genetic variation.

Table 3 Suitablity of two hosts for two populations of *Habrobracon hebetor*

Population[a]	TOA1		JPN	
Host	*Corcyra cephalonica*	*Plodia interpunctella*	*Corcyra cephalonica*	*Plodia interpunctella*
n	10	10	10	10
No. of eggs produced in 7 days[b]	156.2 ± 8.0Aa	82.0 ± 7.3Ba	128.5 ± 14.1Ab	78.9 ± 6.4Ba
Adult emergence[b] (%)	76.4 ± 1.7Aa	63.2 ± 3.6Ba	43.9 ± 3.9Ab	44.5 ± 2.0Ab
Sex ratio[b] (% female)	51.9 ± 6.8Aa	42.4 ± 7.7Ba	64.1 ± 3.8Ab	57.2 ± 3.2Ab

[a]See Table 1 for details of populations (TOA1 from Thailand and JPN from Japan)

[b]Values are mean ± SE. Values in a row followed by the same capital letters do not differ significantly between two hosts, and those followed by the same lowercase letters do not differ significantly between two populations (likelihood ratio Chi-square test, $p > 0.05$)

Our results also showed that Thai populations were genetically different from the Japanese population. The presence of two distinct clades within *H. hebetor* was indicated by both *COI* and 16S sequences (Fig. 1). Based on *COI* sequences, Thai populations belong to the same clade as populations in Spain and India (Fig. 1). Based on 16S sequences, Thai populations belong to the same clade as the population in Barbados, which was originally distributed in India (Heimpel et al. 1997), and populations in Japan belong to the same clade as populations in the USA (Heimpel et al. 1997) (Fig. 1). The pairwise distances between the two clades were ca. 7% for COI and 2% for 16S, indicating that the two clades differed at nearly the species level (Hebert et al. 2003). The strain *H. hebetor* from Thailand also contains insects identified as *H. brevicornis* (Wesmael) (Fig. 1). This is consistent with a previous study that suggested that *H. brevicornis* is considered a junior synonym of *H. hebetor* (Papp 2008; Yu et al. 2012). However, Japanese populations of *H. hebetor* were clearly separated from *H. brevicornis* at nearly the species level (Fig. 1). The taxonomic status of *H. brevicornis* and *H. hebetor* might need further study.

Host records of *H. hebetor* suggest that there are two groups within *H. hebetor*: one has a limited host range that attacks only stored grain pests. This is distributed in Japan and the USA (Brower et al. 1996; Tamura 1994; Watanabe 1933). The other has a wider host range and attacks stored grain pests and outdoor crop pests; it is distributed in the Sahel, Italy, Israel, Iraq, Azerbaijan and India (Al-Maliky and Al-Izzi 1986; Gahukar et al. 1986; Gerling 1971; Loni et al. 2016; Mamedov 1989; Puttarudriah and Basavanna 1956). Our DNA analysis results seem to correspond to these host records. In this scenario, one clade with a narrow host range seems to be distributed to a limited area, and the other one with a wide host range is distributed widely in Eurasia and Africa.

Results of cross tests demonstrated that Thai populations are sexually isolated from the population in Japan. This is consistent with results of the previous study using two clades from the USA and Barbados, showing that there are no mating and production of female offspring from the crosses of different clades (Heimpel et al. 1997). As our cross tests showed sexual isolation between the two clades of Japan and Thailand, there may be sexual isolation between the two clades of Japan/the USA and Africa/Eurasia populations.

The Thai population produced more eggs than the Japanese population (Table 3). This is consistent with the previous study that showed greater egg production in the "Barbados strain," which belongs to the same clade as the Thai population (Fig. 1) (Heimpel et al. 1997). Our results also showed that the Thai population started oviposition earlier than the Japanese population (Fig. 2). Greater egg production and a shorter preoviposition period may be advantageous for the Thai population when they are used as a biologic control agent. Egg production and survival of the Japanese population did not differ between the two host species, but the Thai population produced more eggs on *C. cephalonica* than on *P. interpunctella*, and adult emergence was higher on the former than the latter (Table 3). Many species of Lepidoptera have been reported as potential hosts for rearing of *H. hebetor* (Ghimire and Phillips 2010, 2014; Khalil et al. 2016; Saadat et al. 2014; Youm and Gilstrap 1993). Although further investigation would be required to determine the most suitable host species for mass rearing in Thailand, our results showed that *C. cephalonica* is more suitable for rearing the *H. hebetor* Thai population than *P. interpunctella*.

The sex ratios of Japanese populations were always female biased, while Thai populations showed both female- and male-biased sex ratios (Tables 2, 3). In *H. hebetor*, the sex determination system has been known as complementary sex determination (CSD) in which fertilized eggs develop to diploid males when they are homozygous at the sex locus or females when they are heterozygous and unfertilized eggs develop to haploid males (Whiting 1943). Occurrence of homozygosity is high under inbreeding (Cook and Crozier 1995). The small genetic diversity in Thai populations may indicate that inbreeding occurs in these populations, which leads to a male-biased sex ratio. Survival of diploid males is low in *H. hebetor* in the USA, causing a female-biased sex ratio (Petters and Mettus 1980; Whiting 1943). In the

"Barbados strain" and "*Bracon* sp. near *hebetor*," which are in the same clade as Thai populations, however, it has been reported that survival of diploid males is high, resulting in a male-biased sex ratio (Heimpel et al. 1997; Holloway et al. 1999). These indicate that the male-biased sex ratio in the Thai population in the current study might be explained by the frequent occurrence of diploid males.

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