# *CHARTOCERUS* SP. (HYMENOPTERA: SIGNIPHORIDAE) AND *PACHYNEURON CRASSICULME* (HYMENOPTERA: PTEROMALIDAE) ARE OBLIGATE HYPERPARASITOIDS OF *DIAPHORENCYRTUS ALIGARHENSIS* (HYMENOPTERA: ENCYRTIDAE) AND POSSIBLY *TAMARIXIA RADIATA* (HYMENOPTERA: EULOPHIDAE)

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Supplementary material for this article in Florida Entomologist 97(2) (June 2014) is online at http://purl.fcla.edu/fcla/entomologist/browse

Abstract

Two species of suspected hyperparasitoids, *Chartocerus* sp. and *Pachyneuron crassiculme*, emerged from parasitized *Diaphorina citri* nymphs collected in Punjab Pakistan over 15-22 Apr 2013. Exposure tests conducted in quarantine on *D. citri* nymphs parasitized by *Tamarixia radiata* and *Diaphorencyrtus aligarhensis*, as well as unparasitized *D. citri* nymphs, confirmed that *Chartocerus* sp. and *P. crassiculme* are hyperparasitoids. Both *Chartocerus* sp. and *P. crassiculme* successfully reproduced on *D. aligarhensis*, with one instance of *P. crassiculme* reproducing on *T. radiata*. There was no emergence from unparasitized *D. citri*.

Key Words: choice test, no-choice test, quarantine

Resumen

Dos especies de hiperparasitoides sospechosos, *Chartocerus* sp. y *Pachyneuron crassiculme*, emergieron de ninfas parasitadas de *Diaphorina citri* recolectadas en Punjab Pakistán del 15 al 22 de abril del 2013. Las pruebas de la exposición realizada en cuarentena sobre ninfas de *D. citri* parasitadas por *Tamarixia radiata* y *Diaphorencyrtus aligarhensis*, así como ninfas de *D. citri* no parasitadas confirmaron que *Chartocerus* sp. y *P. crassiculme* son hiperparasitoides. Tanto *Chartocerus* sp. y *P. crassiculme* se reprodujeron con éxito sobre *D. aligarhensis*, con un caso de *P. crassiculme* reproducido sobre *T. radiata*. No hubo emergencia de parasitos de las ninfas de *D. citri* no parasitidas.

Palabras Clave: prueba de opción, prueba de elección, cuarentena

Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), was discovered in California USA in 2008. *D. citri* vectors '*Candidatus* Liberibacter asiaticus', a putative causative agent of huanglongbing (HLB), a lethal disease of citrus (Hoffman et al. 2013; Wang & Trivedi 2013). HLB was detected in California in Mar 2012 (Leavitt 2012). To mitigate the threat posed by *D. citri*-HLB to California's citrus industry, a biological control program using *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) sourced from Pakistan was initiated (Hoddle 2012). *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal) (Hymenoptera: Encyrtidae), a second parasitoid of *D. citri* also collected from Pakistan, is currently in quarantine at the University of California, Riverside (UCR). The purpose of this study was to confirm that *Chartocerus* sp. and *P. crassiculme*, both suspected hyperparasitoids, are not primary parasitoids of *D. citri*.

## Materials and Methods

Parasitized *D. citri* host material returned from Punjab Pakistan to quarantine at UCR (15-22 Apr 2013) yielded previously collected *T. radiata* and *D. aligarhensis*, along with several species of known (*Marietta leopardina* Motschulsky [Hymenoptera: Aphelinidae], *Aprostocetus* (*Aprostocetus*) sp. [Hymenoptera: Eulophidae] [Hoddle et al. 2013]) or suspected (*Chartocerus* sp. [Hymenoptera: Signiphoridae], *Pachyneuron crassiculme* Waterston [Hymenoptera: Pteromalidae] and *Psyllaphycus diaphorinae* [Hymenoptera: Encyrtidae]) hyperparasitoids.

To confirm that *Chartocerus* sp. (Fig. 1A. male, B. female) and *P. crassiculme* (Fig. 2A. male, B. female) are not primary parasitoids of *D. citri*, exposure trials using 10 sets of 4-7 *Chartocerus* sp. and 10 pairs of 1 male and 1 female *P. crassiculme* that emerged from material collected in Pakistan

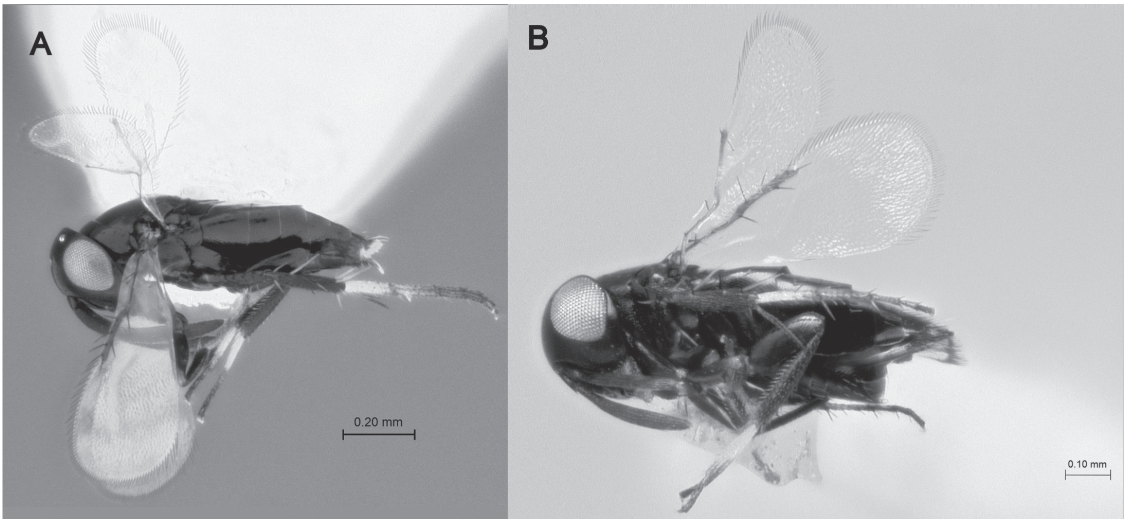


Fig. 1. *Chartocerus* sp. male (A) and female (B). This figure is shown in color in the supplementary document in Florida Entomologist 97(2) (2014) online at http://purl.fcla.edu/fcla/entomologist/browse .

were rotated through each of 4 treatment types between 26 Apr and 24 May, 2013 in quarantine at UCR. It was not possible to reliably sex live *Chartocerus* sp., so this species was exposed in groups (assumed to contain at least 1 female each) unless a pair was otherwise observed mating. Exposure treatments consisted of: (A) nymphs parasitized by *T. radiata* ($n$ = 8 replicates of 5-10 parasitized nymphs for *Chartocerus* sp. and 9 replicates of 5 parasitized nymphs for *P. crassiculme*), 5-9 days post-exposure to *T. radiata*; (B) nymphs parasitized by *D. aligarhensis* ($n$ = 8 replicates of 5-10 parasitized nymphs for *Chartocerus* sp. and 10 replicates of 5 for *P. crassiculme*), 10-14 days post-exposure to *D. aligarhensis*; (C) unparasitized third to fourth instar *D. citri* nymphs ($n$ = 9 replicates of 5-10 unparasitized nymphs for *Chartocerus* sp. and 10 replicates of 5 nymphs for *P. crassiculme*); and (D) each of the 3 previously listed host types (A, B, and C) presented simulta-

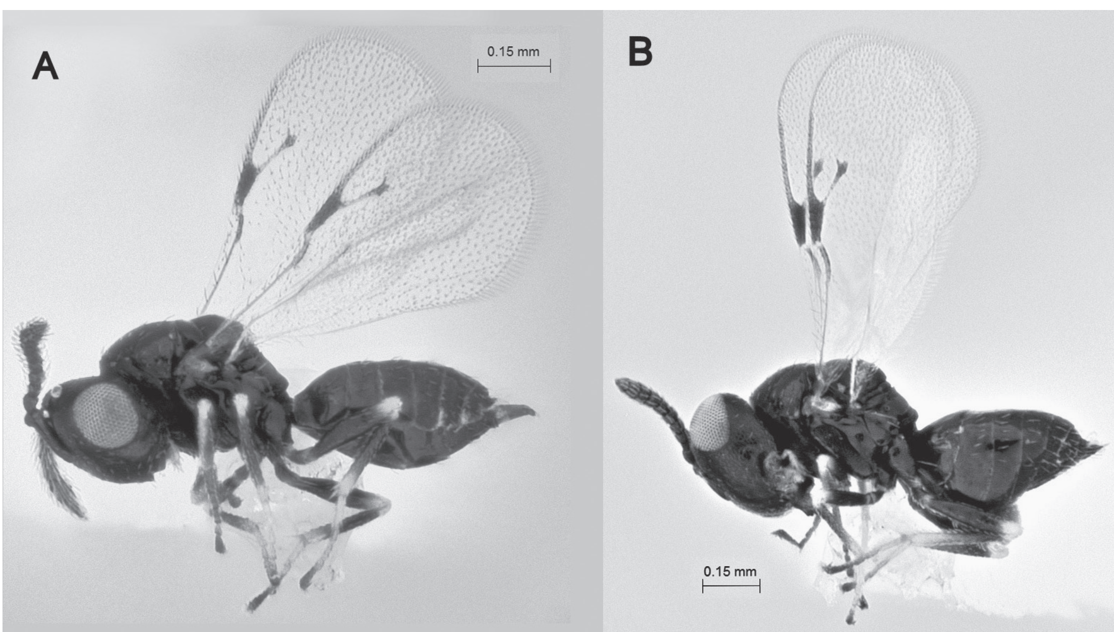


Fig. 2. *Pachyneuron crassiculme* male (A) and female (B). This figure is shown in color in the supplementary document in Florida Entomologist 97(2) (2014) online at http://purl.fcla.edu/fcla/entomologist/browse

neously in a choice cage ($n$ = 9 replicates of 5-10 of each host type for *Chartocerus* sp. and 9 replicates of 5 of each host type for *P. crassiculme*).

Each replicate was comprised of host material for each treatment type exposed to a group of potential hyperparasitoids for 24 h each. Hosts were exposed sequentially in a different order for each replicate to prevent bias due to presentation order. Emergence rates of *T. radiata* ($n$ = 5 parasitized nymphs on each of 10 cuttings) and *D. aligarhensis* ($n$ = 5 parasitized nymphs on each of 10 cuttings) determined baseline mortality for primary parasitoids in the absence of hyperparasitoids. Unparasitized *D. citri* nymphs ($n$ = 5 fourth instar nymphs on each of 10 plants) provided data on nymph mortality in the absence of hyperparasitoids. Mummies of *T. radiata* and *D. aligarhensis* used in exposure experiments were sourced from colonies maintained in quarantine at UCR.

*Diaphorina citri* nymphs parasitized by either *T. radiata* or *D. aligarhensis* for no-choice treatments were presented on small *Citrus volkameriana* cuttings. *Citrus volkameriana* seedlings grown in 114 mL Cone-tainers™ (SC7 Stubby, 3.8 cm diameter, Stewe and Sons Inc., Oregon) and infested with *D. citri* nymphs were used to expose unparasitized *D. citri* nymphs to *Chartocerus* sp. and *P. crassiculme*. Clear plastic vials (Thornton Plastic Co. 148 mL capacity, Salt Lake City, Utah) with three 12 mm diam ventilation holes covered with ultra-fine organza were inverted and placed over the top of the plant and fitted into the corresponding vial lid, which had a hole cut in the center to allow it to be fitted around the cone (Irvin et al. 2009).

Choice treatments were set up in 15 cm × 15.3 cm × 15.3 cm (h × w × d) clear plastic boxes (S&W Plastics, Riverside, California) with a 30 cm sleeve sewn from no-see-um netting (Skeeta Mosquito & Other Insect Protection Products, Bradenton, Florida). Unparasitized *D. citri* nymphs in Cone-tainers and *T. radiata*- and *D. aligarhensis*-parasitized nymphs on *C. volkameriana* cuttings in water were placed in the cage without ventilated vials on top to allow free access to all 3 host types simultaneously. After 24 h, each host type was enclosed with an inverted ventilated vial to contain all insects that emerged from each host type. All experiments were conducted in quarantine at UCR's Insectary and Quarantine facility, at 27 °C, 50% RH, and 14:10 h L:D. Replicates were observed daily after initial exposure, and total numbers of each emerged species were recorded per treatment.

## RESULTS

No-choice treatments resulted in *Chartocerus* sp. reproducing successfully only on *D. aligarhensis* (Table 1). Mean emergence time for *Char-*

TABLE 1. EMERGENCE AND MORTALITY RATES FOR *CHARTOCERUS* SP. EXPOSED TO UNPARASITIZED THIRD AND FOURTH INSTAR *D. CITRI* NYMPHS, AND NYMPHS PARASITIZED BY *T. RADIATA*, AND *D. ALIGARHENSIS* IN NO-CHOICE AND CHOICE TREATMENTS.

| | No-choice | | | | | Choice | | | |
|---|---|---|---|---|---|---|---|---|---|
| Host | Total No. Exposed | % Host Emergence | % Parasitism | % Dead[5] | % Missing[6] | Total No. Exposed | % Host Emergence | % Dead[5] | % Missing[6] |
| *D. citri* | 65 | 72.31%[1] | 0.00% | 9.23% | 18.46% | 65 | 81.54%[1] | 13.85% | 4.61% |
| *T. radiata* | 67 | 67.16%[2] | 0.00% | 26.87% | 5.97% | 62 | 66.13%[2] | 25.81% | 8.06% |
| *D. aligarhensis* | 60 | 33.33%[3] | 46.67%[4] | 16.67% | 3.33% | 65 | 53.85%[3] | 29.23% | 16.92% |

[1]Percentage of *D. citri* adults that emerged from unparasitized nymphs.
[2]Percentage of *T. radiata* adults that emerged from parasitized nymphs.
[3]Percentage of *D. aligarhensis* adults that emerged from parasitized nymphs
[4]Percentage of hosts killed by parasitism. Actual number of host killed = 28; actual number of *Chartocerus* sp. adults emerged = 33, demonstrating 2 observed instances of superparasitism.
[5]Percentage of hosts found dead.
[6]Percentage of hosts unaccounted for at time of data collection.

*tocerus* sp. offspring from *D. aligarhensis* was 18.36 days ± 2.34 (SE). *Pachyneuron crassiculme* produced progeny on *D. aligarhensis* and *T. radiata* in no-choice treatments, though parasitism was much higher on *D. aligarhensis* (Table 2). Mean emergence times for males and females were 12.83 days ± 2.48 (SE) and 11.33 days ± 2.05 (SE), respectively. *Pachyneuron crassiculme* had a single male emerge from *T. radiata* after 11 days. Emergence rates for control treatments of *T. radiata*, *D. aligarhensis*, and *D. citri* were 84%, 88%, and 88%, respectively (Table 3). *Chartocerus* sp. and *P. crassiculme* failed to reproduce on unparasitized *D. citri* nymphs.

Immature *D. aligarhensis* exposed to *Chartocerus* sp. in no-choice tests experienced 47% parasitism, 17% died from undetermined causes, 3% were unaccounted for, and 33% emerged as adult *D. aligarhensis*. In 20% of trials (i.e., 2 of 10 replicates) *Chartocerus* sp. exhibited superparasitism, with 11 adults emerging from 9 *D. aligarhensis* mummies in 1 replicate, and 6 adults emerging from 3 mummies in the second. In no-choice tests, immature *T. radiata* exposed to *Chartocerus* sp. exhibited 0% parasitism, 27% of mummies died from unknown causes, 6% disappeared, and 67% emerged as adult *T. radiata*.

In no-choice tests where *P. crassiculme* was exposed to immature *D. aligarhensis*, 28% of hosts were parasitized by *P. crassiculme*, 19% died from unknown causes, and 53% emerged as adult *D. aligarhensis*. On *T. radiata*, *P. crassiculme* successfully parasitized only 2% of host material (i.e., one host), 40% died from unknown causes, 7% were unaccounted for, and 51% emerged as adult *T. radiata*. Unknown mortality may be attributable to superparasitism, host feeding, or a combination of both by *P. crassiculme*.

There was no successful parasitism of any host in choice tests for either *Chartocerus* sp. or *P. crassiculme*. However, elevated mortality rates were observed for *T. radiata* (26% when exposed to *Chartocerus* sp.; 28% for *P. crassiculme*) and *D. aligarhensis* (29%; 13%). In comparison, control mortality for *T. radiata* and *D. aligarhensis* were < 13% in the absence of these hyperparasitoids. When viewed collectively, data from exposure trials demonstrates that *Chartocerus* sp. and *P. crassiculme* are obligate hyperparasitoids within the *D. citri-Tamarixia-Diaphorencyrtus* system. Immediately following the conclusion of trials, all *Chartocerus* sp. and *P. crassiculme* material was killed in quarantine and preserved in 95% ethanol. Voucher specimens were deposited in the Entomology Museum at UCR (Table 4).

Assuming *Chartocerus* sp. and *P. crassiculme* preferentially parasitize *D. aligarhensis* as these exposure trial data suggest, the frequency of *Chartocerus* sp. and *P. crassiculme* emergence in quarantine from material collected from Punjab Pakistan in April 2013 was significant in compar-

TABLE 2. EMERGENCE AND MORTALITY RATES FOR *PACHYNEURON CRASSICULME* EXPOSED TO UNPARASITIZED THIRD AND FOURTH INSTAR *D. CITRI* NYMPHS, AND NYMPHS PARASITIZED BY *T. RADIATA* AND *D. ALIGARHENSIS* IN NO-CHOICE AND CHOICE TREATMENTS.

| | No-choice | | | | | Choice | | | |
|---|---|---|---|---|---|---|---|---|---|
| Host | Total No. Exposed | % Host Emergence | % Parasitism[4] | % Dead[5] | % Missing[6] | Total No. Exposed | % Host Emergence | % Dead[5] | % Missing[6] |
| *D. citri* | 50 | 68.00%[1] | 0.00% | 24.00% | 8.00% | 45 | 73.33%[1] | 13.33% | 13.33% |
| *T. radiata* | 45 | 51.11%[2] | 2.22% | 40.00% | 6.67% | 46 | 71.74%[2] | 28.26% | 0.00% |
| *D. aligarhensis* | 53 | 52.83%[3] | 28.30% | 18.87% | 0.00% | 46 | 86.96%[3] | 13.04% | 0.00% |

[1]Percentage of *D. citri* adults that emerged from unparasitized nymphs.
[2]Percentage of *T. radiata* adults that emerged from parasitized nymphs.
[3]Percentage of *D. aligarhensis* adults that emerged from parasitized nymphs.
[4]Percentage of *Pachyneuron crassiculme* adults that successfully emerged from parasitized hosts.
[5]Percentage of hosts found dead.
[6]Percentage of hosts unaccounted for at time of data collection.

TABLE 3. EMERGENCE RATES OF UNPARASITIZED THIRD AND FOURTH INSTAR *D. CITRI* NYMPHS AND NYMPHS PARASITIZED BY *T. RADIATA* AND *D. ALIGARHENSIS* IN CONTROL TREATMENTS NOT EXPOSED TO HYPERPARASITOIDS.

| Host | Total No. Exposed | No. Adults Emerged | No. Dead Hosts[4] | No. Missing Hosts[5] |
|---|---|---|---|---|
| *D. citri* | 50 | 44[1] | 1 | 0 |
| *T. radiata* | 50 | 42[2] | 6 | 2 |
| *D. aligarhensis* | 52 | 46[3] | 3 | 1 |

[1]Total number of *D. citri* adults that matured from unparasitized nymphs.
[2]Total number of *T. radiata* adults that emerged from parasitized nymphs.
[3]Total number of *D. aligarhensis* adults that emerged from parasitized nymphs.
[4]Total number of hosts found dead.
[5]Total number of hosts unaccounted for at time of data collection.

TABLE 4. SPECIMEN ACCESSION NUMBERS FOR ALL SPECIES USED IN EXPOSURE TRIALS AND DEPOSITED IN THE ENTOMOLOGY MUSEUM AT THE UNIVERSITY OF CALIFORNIA RIVERSIDE.

| Species | Accession No. |
|---|---|
| *D. citri*[1] | UCRC_ENT00334428 |
| *T. radiata*[2] | UCRC_ENT00334402-334418 |
| *D. aligarhensis*[2] | UCRC_ENT00334426-334427 |
| *Chartocerus* sp.[2] | UCRC_ENT00417173-00417182 |
| *P. crassiculme*[2] | UCRC_ENT00417183-00417187 |

[1]Multiple individuals of Pakistani *D. citri* preserved in a single vial of 95% ethanol.
[2]Point-mounted individuals

ison to *D. aligarhensis* emergence rates. *Chartocerus* sp. (237 individuals reared), *P. crassiculme* (181), and *D. aligarhensis* (743) represented 20%, 16%, and 64% of material reared, respectively, within this complex. A total of 292 *T. radiata* were reared from April 2013 collections. Exposure trials suggest that the lower numbers of *T. radiata* obtained from Pakistan in April 2013 were not likely due to hyperparasitism.

## ACKNOWLEDGMENTS

The authors would like to thank Roger Burks (UCR) for assistance with specimen mounting, photography, and identification. Serguei Triapitsyn (UCR) helped with specimen identification and deposition of voucher specimens. Anamaria Dal Molin (TAMU) identified *Chartocerus* sp. Funding for this project was provided, in part, by funds issued by the California Department of Food and Agriculture's Specialty Crops Program and the California Citrus Research Board.

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