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HOW MANY PRIMARY PARASITOID SPECIES ATTACK NYMPHS OF *DIAPHORINA CITRI* (HEMIPTERA: LIVIIDAE) IN PUNJAB, PAKISTAN?

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Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is the vector of a bacterium, '*Candidatus* Liberibacter asiaticus' (*C*Las), that causes a lethal citrus disease, huanglongbing. This insect was first detected in California (USA) 2008 and *C*Las was discovered in residential citrus in 2012 (Kumagai et al. 2013). ACP is the target of a classical biological control program in California with host specific parasitoids sourced from Punjab Pakistan (Hoddle 2010, 2012; Hoddle & Hoddle 2013; Hoddle & Pandey 2014). Foreign exploration for natural enemies has focused on Punjab Pakistan because this is likely part of the native range of ACP (Halbert & Manjunath 2004; Hussain & Nath 1927) and natural enemy diversity is consequently expected to be highest here. Further, Punjab has ≈ 70% climate match with important citrus producing areas in California, which should benefit biological control efforts because introduced natural enemies would be pre-adapted to prevailing climates in the new range (Hoddle 2012; Hoddle & Hoddle 2013). Hussain & Nath (1927) hinted at a rich parasitoid fauna associated with ACP nymphs in Punjab Pakistan, with up to 9 species of chalcidoids being reared from field collected material. This guild of natural enemies is stated to be more important than predators in regulating ACP populations in Pakistan even though it is subject to hyperparasitism (Hussain & Nath 1927). Of these nymphal parasitoids, just one, the most commonly collected species was named, this being *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) (referred to as *Tetrastichus radiatus* [Waterston 1922] by Hussain & Nath [1927]). A second parasitoid species attacking ACP nymphs, *Diaphorencyrtus aligarhensis* (Shafee, Alam and Agarwal) (Hymenoptera: Encyrtidae), was described from India (Shafee et al. 1975) and it has been documented as an ACP natural enemy in other countries as well (Halbert & Manjunath 2004), including the same general areas in Punjab Pakistan where Hussain & Nath (1927) did their original field work (Khan et al., 2014). Therefore, of the 9 primary parasitoid species that Hussain & Nath (1927) state attack ACP nymphs in Punjab Pakistan, 2 are known, *T. radiata* and *D. aligarhensis*, but the identities of the remaining 7 species are unknown. Foreign exploration efforts for parasitoids associated with ACP nymphs over the period Apr 2011 to Apr 2013 provided an opportunity to determine the identities of these 7 additional species alluded to by Hussain & Nath (1927).

Field collections of ACP nymphs for the rearing of parasitoids were made in Punjab Pakistan over 10 Mar to 11 Apr 2011, 23 May to 13 Jun 2011, 14 Oct to 4 Nov. 2011, 19 May to 5 Jun 2012, and 1-22 Apr 2013. A total of 173 site visits were made to 47 different locations; Faisalabad (this location is Lyallpur in Hussain & Nath [1927]), 127 collections were made from 8 sites, all on research properties associated with the University of Agriculture Faisalabad, of which one site, PARS is 12 km from the main campus; Toba Tek Singh, 21 collections were made from 20 sites; Sargodha, 22 collections were made from 17 sites; Bhalwal, 1 collection from 1 site; Lalian, 2 collections made from 1 site. Different citrus species or citrus relatives (all Rutaceae) were sampled for ACP nymphs, which included in descending order of sampling intensity, kinnow (*Citrus reticulata* Blanco), sweet orange (*C. sinensis* L. Osbeck), grapefruit (*C.* x *paradise* MacFadyen), sweet orange jasmine (*Murraya exotica* L.), and limes (*C. aurantiifolia* Swingle). ACP females only oviposit on flush growth and colonies of nymphs develop exclusively on this young plant material (Hall et al. 2008). Consequently, flush growth infested with ACP nymphs was harvested from field sites, labeled, and placed into plastic cups with water. Location (with GPS coordinates), date of sampling, and host plant from which samples were taken were recorded. Depending on the amount of material collected from each site, labeled flush bouquets (uninfested foliage was removed from cut stems in the field) were maintained in plastic cups holding water and each bouquet was covered with a clear plastic soda bottle that had had the bottom removed. These labeled "bottle cages" trapped parasitoids emerging from individual bouquets. Bottle cages were stored inside BugDorm-2120F insect rearing tents (60 × 60 × 60 cm [MegaView Science Company Ltd, Taichung, Taiwan]) and held in the laboratory at room temperature until parasitoids emerged and foliage had completely deteriorated. If sufficient material was collected from one site to fill a BugDorm, these bouquets were not enclosed in bottle

cages and parasitoids and adult ACP emerged directly inside insect tents. Parasitoids were either aspirated from bottle cages or BugDorms into clear plastic vials or captured on walls by placing a clear plastic 2.0 mL centrifuge vials over the parasitoid. All vials with captured parasitoids were labeled with locality data. When ACP mummies were abundant on collected plant material, they were excised either individually or in small groups on the smallest amount of plant material possible and placed into ventilated plastic vials (22 mm diam; 40 mm height) for emergence and labeled by collecting site and date. All vials were provisioned with honey droplets for parasitoids to feed on. Parasitoids that died prior to movement from Pakistan to the Insectary and Quarantine Facility at the University of California Riverside (I-&-Q-UCR) were collected and preserved in 95% ethanol in labeled vials for identification. Live parasitoids, ACP mummies, or late instar ACP nymphs that were potentially parasitized but attached to foliage were moved from Pakistan to I-&-Q-UCR under USDA-APHIS permit 526P-11-00103. In quarantine, live parasitoids were identified to species when possible (e.g., *T. radiata* and *D. aligarhensis*), unknown parasitoids of similar appearance were grouped as morphotypes, 1-2 were slide mounted for identification, and if the initial identification suggested they could be potential parasitoids of ACP nymphs (e.g., *Psyllaphycus diaphorinae* Hayat [Hymenoptera: Encyrtidae] [Triapitsyn et al. 2013]) attempts were made to establish colonies by exposing adult parasitoids to mixed stage ACP populations on caged *Citrus volkameriana* in quarantine. Suspected hyperparasitoids (see Table 1) were exposed to ACP nymphs parasitized by *T. radiata* or *D. aligarhensis* that were maintained in quarantine colonies.

A total of 3,675 parasitoids were reared in I-&-Q-UCR from ACP collections in Punjab Pakistan. Reared specimens represented at least 13 species in 13 genera (Table 1). Voucher specimens of parasitoids reared from this study are preserved in the Entomology Research Museum, University of California at Riverside (UCRC).

Two primary parasitoids, *T. radiata* (55% of collected specimens) and *D. aligarhensis* (28% of collected material) were reared from ACP nymphs. Five species, *Marietta leopardina* Motschulsky (Hymenoptera: Aphelinidae), *Aprostocetus* (*Aprostocetus*) sp. (Hymenoptera: Eulophidae), *Chartocerus* sp. (Hymenoptera: Signiphoridae), *Pachyneuron crassiculme* Waterston (Hymenoptera: Pteromalidae), and *Psyllaphycus diaphorinae* were demonstrated in quarantine to be hyperparasitoids of *T. radiata* and *D. aligarhensis* and were unable to develop as primary parasitoids on

TABLE 1. PARASITOID SPECIES REARED FROM COLLECTIONS OF *DIAPHORINA CITRI* NYMPHS IN PUNJAB PAKISTAN.

Parasitoid Species Collected	Collection Date					
	10 Mar – 11 Apr 2011	23 May – 13 June 2011	14 Oct – 4 Nov 2011	19 May – 5 Jun 2012	1 – 22 Apr 2013	Total Collected
[1]*T. radiata*	80	406	1,1012	238	292	2,028
[2]*D. aligarhensis*	70	25	2	164	744	1,023
[3]*Aprostocetus* (*Aprostocetus*) sp.	0	0	0	3	11	14
[4]*Chartocerus* sp.	0	0	0	0	224	224
[5]*Cirrospilus* sp.	5	0	1	0	2	8
[5]*Citrostichus phyllocnistoides*	16	0	3	3	0	22
[6]*Encarsia* sp.	0	0	13	1	0	14
[7]*Erythmelus panis*	0	0	0	1	0	1
[8]*Gonatocerus* sp.	0	1	0	0	0	1
[9]*Marietta leopardina*	0	0	52	24	0	76
[10]*Psyllaphycus diaphorinae*	0	0	22	1	8	31
[11]*Pachyneuron crassiculme*	0	0	0	0	182	182
[5]*Sympiesis* sp.	54	4	0	0	0	58
Unidentified spp.	0	0	14	2	32	48

[1]and [2]are eulophid and encyrtid primary parasitoids, respectively, attacking *D. citri* nymphs
[3]Eulophid hyperparasitoid attacking *T. radiata* and *D. aligarhensis*
[4]Signiphorid hyperparasitoid attacking *D. aligarhensis*
[5]Eulophid parasitoids attacking leafminer larvae
[6]Aphelinid parasitoid attacking scales
[7]Mymarid parasitoid attacking tingid eggs
[8]Mymarid parasitoid attacking cicadellid eggs
[9]Aphelinid hyperparasitoid attacking *D. aligarhensis* and *T. radiata*
[10]Encyrtid hyperparasitoid attacking *D. aligarhensis*
[11]Pteromalid hyperparasitoid attacking *D. aligarhensis*
[12]Unidentified parasitoids from unknown hosts

unparasitized ACP nymphs (Hoddle et al. 2013; Bistline-East & Hoddle 2014). An additional 6 parasitoid species were reared from plant material, either bouquets, or excised nymphs or mummies attached to very small pieces of twig or leaf at I-&Q-UCR. These parasitoids were identified to species where possible, and identifications were used to determine likely host associations (e.g., *Erythmelus panis* [Enock] and *Gonatocerus* sp. [both Hymenoptera: Mymaridae] are parasitoids of lace bug (Hemiptera: Tingidae) and leafhopper (Hemiptera: Cicadellidae) eggs, respectively [Table 1]). None of these natural enemies were considered likely parasitoids of ACP nymphs (Table 1).

The results of these surveys and longer-term phenology studies on ACP in Faisalabad Pakistan (Khan et al. 2014) strongly suggest that there are only 2 primary parasitoid species associated with ACP nymphs in Punjab Pakistan and these natural enemies are likely more important than predators in regulating ACP populations (Khan et al. 2014), a finding which supports Hussain & Nath's (1927) observations. The 9 species alluded to by Hussain & Nath (1927) were likely a combination of hyperparasitoids (mentioned but not named by Hussian & Nath [1927]) attacking immature *T. radiata* and *D. aligarhensis*; parasitoids attacking other unobserved insects co-inhabiting ACP infested foliage, (e.g., the leafminer parasitoid *Citrostichus phyllocnistoides* [Narayanan] [Hymenoptera: Eulophidae]) or parasitoids with pronounced sexual dimorphism such as some species of encyrtids (e.g., *P. diaphorinae*) of which males and females could have been assumed to be different species. Parasitoids attacking insects other than ACP nymphs were most commonly collected during Mar-Apr 2011 and May-Jun 2011 when material returned to UCR-I-&-Q was associated with excess plant material (e.g., parts of small cut leaves attached to ACP infested twigs). Excision of ACP mummies and large nymphs from live plant material over Oct-Nov 2011, May-Jun 2012, and Apr 2013 minimized greatly excess plant material and reduced the collection of unwanted parasitoids associated with ACP infested foliage (Table 1). Additionally, there appeared to be significant temporal variability associated with the collection of hyperparasitoids attacking *T. radiata* and *D. aligarhensis* and no single species was collected consistently (Table 1).

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Summary

Hussain & Nath (1927) stated that 9 species of parasitoids attack nymphs of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) in Punjab Province of Pakistan. Foreign exploration for natural enemies of *D. citri* over Mar 2011 to Mar 2013 in Punjab Pakistan resulted in the collection of 3,675 parasitoids representing at least 13 different species. However, just 2 species of primary parasitoids, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) (55% of collected specimens) and *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae) (28% of collected material) attacked *D. citri* nymphs and 5 species of hyperparasitoids attacked the immature stages of these two primary parasitoids. The remaining 11 parasitoid species that were identified likely attacked unnoticed insect species that co-infested foliage with *D. citri* nymphs. We conclude that Hussain & Nath (1927) overestimated the number of primary parasitoids attacking *D. citri* nymphs in Punjab Pakistan.

Key Words: foreign exploration, host rearing, quarantine

Resumen

Hussain y Nath (1927) reportan 9 especies de parasitoides atacando ninfas de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) en la provincia de Punjab en Pakistán. Exploración del exterior para los enemigos naturales de *D. citri* de marzo 2011 hasta marzo 2013 en Punjab Pakistán resultó en una colección de 3675 parasitoides que representen, por lo menos, 13 especies diferentes. Sin embargo, sólo 2 especies de parasitoides primarios, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) (55% de los especimenes recolectados) y *Diaphorencyrtus aligarhensis* (Shafee, Alam y Agarwal) (Hymenoptera: Encyrtidae) (28% de los especimenes recolectados) atacan ninfas de *D. citri* y 5 especies de hiperparasitoides atacan los estadios inmaduros de estos dos parasitoides primarios. El resto de las especies de parasitoides probablemente atacan especies de insectos imperceptibles que estan en el follaje con ninfas de *D. citri*. Llegamos a la conclusión de que Hussain y Nath (1927) sobreestimaron el número de parasitoides primarios que atacan ninfas *D. citri* en Punjab Pakistán.

Palabras Clave: exploración extranjera, cría de hospedero, cuarentena

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