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Host Range Testing of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) for Use in Classical Biological Control of *Diaphorina citri* (Hemiptera: Liviidae) in California

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ABSTRACT Host range tests for *Diaphorencyrtus aligarhensis* (Shafee, Alam, & Agarwal) (Hymenoptera: Encyrtidae), an endoparasitoid of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), sourced from Punjab Pakistan, were conducted in quarantine at the University of California, Riverside, CA. Seven nontarget psyllid species representing four psyllid families were exposed to mated *D. aligarhensis* females in four different treatment types: 1) short sequential no-choice treatments, 2) prolonged sequential no-choice treatments, 3) prolonged no-choice static treatments, and 4) choice treatments. Selection of nontarget psyllid species was based on phylogenetic proximity to *D. citri*, likelihood of being encountered by *D. aligarhensis* in the prospective release areas in California, and psyllid species in biological control of invasive weeds. *D. aligarhensis* exhibited high host affinity to *D. citri*, and only parasitized one nontarget species, the pestiferous potato psyllid, *Bactericera cockerelli* (Sulc), at low levels (<14%). Based on the results of this study, we conclude that *D. aligarhensis* has a narrow host range and exhibits a high level of host specificity, as it shows a significant attack preference for the target pest, *D. citri*. Results presented here suggest *D. aligarhensis* poses minimal risk to nontarget psyllid species in California.

KEY WORDS biological control, *Diaphorencyrtus aligarhensis*, *Diaphorina citri*, host range testing, huanglongbing

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), an invasive pest of citrus, was discovered in southern California in August 2008 (Grafton-Cardwell 2010, Gutierrez and Ponti 2013). While psyllid feeding can damage citrus flush, the most critical threat presented by *D. citri* is its ability to transmit the bacterium *Candidatus* Liberibacter asiaticus Jagoueix, Bové, & Garnier (*C*Las), a phloem-limited fastidious bacterium (α-proteobacteria), which is recognized as a causative agent of huanglongbing (HLB), a lethal disease in citrus (Hall et al. 2013). Both nymphal and adult psyllids can become infected with *C*Las when feeding on infected trees (Halbert and Manjunath 2004). Adult *D. citri* vector HLB as they disperse and feed, transmitting the bacterium within salivary excretions into phloem tissue (Bové 2006). Trees infected with HLB suffer foliar dieback, mottled yellow leaves, and reduced fruit yield ranging from 30 to 100% (Wang and Trivedi 2013). Tree death usually occurs within 8 yr of HLB contraction, though trees may be asymptomatic for several years, making HLB difficult to diagnose in its early stages (Halbert and Manjunath 2004).

The detrimental effects of *D. citri*–HLB are well-known from many commercial citrus-growing areas in the world, particularly in Florida, where HLB was first detected in 2005 (Bové 2006), and had caused an estimated US$1.7 billion in damage by 2012 (Hodges and Spreen 2012). In April 2012, the first HLB-positive tree was discovered in California (Hacienda Heights, Los Angeles County; Kumagai et al. 2013), raising serious concerns for California's citrus industry, which, in 2009, was worth ~US$3 billion, and supported >26,000 jobs (Richards et al. 2014).

Many of California's commercial citrus orchards are in close proximity to urban areas, which can serve as reservoirs from which *D. citri* can migrate into production areas. These residential areas also provide corridors for *D. citri* spread, which makes containment and pesticide treatments difficult (Richards et al. 2014). Insecticide applications to suppress *D. citri* populations in residential areas have been costly; Hoddle and Pandey (2014) estimated that ~6% of housing lots in Los Angeles County expected to have citrus were treated chemically at an overall cost of US$4.7 million, or ~US$100 per residence. Because of this cost and

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difficulty of accessing properties, urban spray programs for suppressing *D. citri* in southern California have been largely abandoned in favor of classical biological control using host-specific parasitoids from *D. citri*'s home range (Hoddle and Pandey 2014).

D. citri has two widely recognized natural enemies that likely coevolved with *D. citri* on the Indian subcontinent: *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam, & Agarwal) (Hymenoptera: Encyrtidae) (Halbert and Manjunath 2004). In an effort to establish a *D. citri* biological control program in California, six foreign exploration trips to Punjab, Pakistan were conducted between September 2010 and April 2013 to search for both known (*T. radiata*, *D. aligarhensis*) and unknown natural enemies. This was accomplished by collecting parasitized *D. citri* mummies and returning them under federal and state issued permits to quarantine at the University of California, Riverside (UCR). Adult parasitoids were collected as they emerged from this material. Punjab, Pakistan, was selected for sourcing natural enemies because it is likely part of the native range of *D. citri* and this area has ~70% climate match to major citrus production areas in California. Good climate match is assumed to be an important factor affecting natural enemy establishment in a new region (Hoddle 2012).

T. radiata has been widely used for the biological control of *D. citri* (Halbert and Manjunath 2004), providing varying levels of control in different areas where is has been introduced, including Réunion Island (excellent control) and Florida (modest control; Aubert and Quilici 1984, Chien et al. 1989, Qureshi and Stansly 2009). Since its approval for release in December 2011, >700,000 *T. radiata* have been released at >600 sites across southern California. Recovery of *T. radiata* at 107 different sites suggests establishment of *T. radiata* is likely (Simmons et al. 2013).

It is anticipated that *D. aligarhensis* could complement *T. radiata* in California in much the same way it is observed in its native range, thereby enhancing *D. citri* biological control. *D. aligarhensis* is an arrhenotokous endoparasitoid, which preferentially parasitizes second- and third-instar *D. citri* (compared with *T. radiata*, an ectoparasitoid, which preferentially parasitizes fourth and fifth instars; Sule et al. 2014). In addition to parasitization, females can kill *D. citri* nymphs via host feeding (Rohrig et al. 2011). *D. aligarhensis* has been used against *D. citri* in various citrus-growing regions including Taiwan, Réunion Island, Saudi Arabia, and Florida (Chien et al. 1989, Al-Ghamdi and Faragalla 2000, Rohrig et al. 2012), where it has provided low levels of *D. citri* control. Florida populations of *D. aligarhensis*, sourced from Taiwan and China, have failed to establish, despite repeated release efforts (Rohrig et al. 2012).

Though *D. aligarhensis* is reported to have no other hosts besides *D. citri* in its native range (Aubert and Quilici 1984, Skelley and Hoy 2004), it was required to undergo host range testing in quarantine in California before release permits would be issued by the U.S. Department of Agriculture–Animal and Plant Health Inspection Service (USDA-APHIS). Determining the potential host range of arthropod biological control agents is important to avoid undesired nontarget effects that may occur as a result of introducing a novel species into a new area (Babendreier et al. 2006). However, selecting nontarget species when faced with the potential of hundreds of subjects and performing host range experiments within a quarantine facility is challenging. It is common practice to test nontarget species that are taxonomically closely related to the target species, as they may most likely be targeted by the natural enemy of interest. Other considerations when selecting nontarget species for host range testing include ecological or niche similarities, potential geographic overlap, and morphological similarities between target and nontarget species. Distantly related species for inclusion in host range testing may be warranted if the nontarget species of concern is a beneficial or endangered species (Kuhlmann et al. 2006).

Methods of testing nontarget species to determine the host range of natural enemies are variable, but commonly entail small-scale laboratory experiments designed to determine attack likelihood (i.e., host range) and preference (i.e., host specificity) of the natural enemy in the presence and absence of target and nontarget species (van Lenteren et al. 2006). To determine the host range and host specificity of *D. aligarhensis*, variations on choice and no-choice exposure tests using the target pest (i.e., *D. citri*) and seven nontarget psyllid (NTP) species were conducted in quarantine at the UCR. The results of these exposure trials are presented here.

Materials and Methods

Selection of NPT Species. California has a rich psyllid fauna, with 164 species represented in 34 genera and 4 families (Percy et al. 2012). Because of this high diversity, a subset of potential NTP hosts was tested. A key concept in host range testing is that native species that are taxonomically closely related to the target host are likely at the highest risk of experiencing attacks from the potential biological control agent (Kuhlmann et al. 2006). However, in California, there are no species in the genus *Diaphorina* or the tribe Diaphorinini, to which *D. citri* belongs, so additional selection criteria were considered for candidate selection. Candidate species were selected for host range testing using the following criteria: 1) NTP taxonomic relatedness to the target host, *D. citri*, 2) likelihood of *D. aligarhensis* encountering the NTP in wilderness areas in close proximity to citrus orchards or backyard gardens, and 3) psyllids used as biological control agents of invasive plants. The species selected for host range testing and their selection criteria are listed in Table 1.

Source of Insects Used in Experiments. *D. citri* used for host specificity experiments were sourced from colonies maintained in the Insectary and Quarantine Facility (IQF) at UCR. *D. citri* were reared on *Citrus volkameriana* V. Ten. & Pasq. and were initiated

Table 1. Psyllid species and selection criteria for host range testing of *D. aligarhensis*

Psyllid species (Family: tribe)	Qualifying criteria for selection				Host plant species used for testing	Source of psyllids/collection site
	Target species	Close taxonomic relatedness	Likely to be encountered in nature	Biological control agent of a weed		
Diaphorina citri Kuwayama (Liviidae: Euphyllurinae)	X				*Citrus volkameriana* V. Ten. & Pasq.[a]	UCR IQF colony
Bactericera cockerelli (Sulc) (Triozidae)			X		*Capsicum annum* L.[a]	Trumble Lab (Dept. of Entomology, UCR)
Heteropsylla sp. (Psyllidae: Ciriacreminae)			X		*Acacia farnesiana* (L.) Willderman[a]	UCR Botanic Gardens, Riverside, CA
Arytainilla spartiophylla (Forester) (Psyllidae: Psyllinae)				X	*Cytisus scoparius* (L.) Link[b]	El Dorado Co., CA
Euphyllura olivina (Costa) (Liviidae: Euphyllurinae)		X	X		*Olea europaea* L.[a]	Murrietta, CA
Heteropsylla texana Crawford (Psyllidae: Ciriacreminae)			X		*Prosopis glandulosa* Torrey[a]	UCR Botanic Gardens, Riverside, CA
Diclidophlebia fremontiae (Khver) (Liviidae: Liviinae)		X			*Fremontodendron californicum* (Torrey) Coville[c]	Big Bear, CA
Boreioglycaspis melaleucae Moore (Aphalaridae)				X	*Melaleuca quinquenervia* (Cav.) S.T. Blake[c]	USDA-ARS- IPRL Ft. Lauderdale, FL

[a] Nontarget psyllid (NTP) nymphs presented on seedlings in Cone-tainers.
[b] Seedlings used in experiments were young wild-collected plants transplanted into Cone-tainers in UCR IQF.
[c] NTP nymphs presented on seedlings in D40 containers.

from southern California-collected *D. citri* adults, which were tested molecularly and confirmed to be HLB-free. NTP species used in host range testing were collected primarily from local wild populations around California and used to found colonies in IQF (Table 1), the exception being *Boreioglycaspis melaleucae* Moore, a weed biocontrol agent targeting *Melaleuca quinquenervia* (Cav.) S. T. Blake, a weedy tree infesting the Florida Everglades. These NTP were shipped to IQF from U.S. Department of Agriculture–Agricultural Research Service–Invasive Plant Research Lab (USDA-ARS-IPRL; Ft. Lauderdale, FL) under USDA permit number P526P-13-02516 and used to found colonies on *M. quinquenervia*.

D. aligarhensis adults were taken from colonies established in UCR IQF, which were originally founded with bi-parental individuals collected from parasitized *D. citri* in Punjab, Pakistan, and shipped to UCR IQF under USDA-APHIS permit number P526P-11-00103. All *D. aligarhensis* females used in trials were between 3 and 14 d old, and were assumed to have mated after being exposed to male *D. aligarhensis* for at least 24 h.

Maintenance of *D. citri* Colonies. *C. volkameriana* used for maintaining *D. citri* colonies were obtained as rooted seedlings <2 yr of age either directly from Willits and Newcomb Inc. citrus nursery (Arvin, CA) or from the California Department of Food and Agriculture (CDFA) rearing facility at the Mt. Rubidoux Field Station (Rubidoux, Riverside, CA). All *C. volkameriana* were grown in 10.16-cm-diameter pots with modified UCR type III potting soil mix. Seedlings were maintained in greenhouses (27 ± 2°C, 50% relative humidity [RH], and natural day length) at UCR Agricultural Operations (AgOps), with daily watering and Osmocote Pro granular smart-release fertilizer (The Scotts Company LLC, Marysville, OH) applied approximately every 3 mo.

D. citri females oviposit only on very young citrus flush (Hall et al. 2008). To promote flush growth, *C. volkameriana* were subjected to regular pruning. Approximately 10–12 d after pruning, plants developed suitable flush for *D. citri* oviposition and were moved from AgOps to IQF for use in *D. citri* colonies. In IQF, *C. volkameriana* were pruned to remove foliage unsuitable for *D. citri* oviposition and a nylon stocking sleeve was fitted over the soil to prevent emergence of soil-borne insects. Plants were then placed into a primary colony cage, constructed using two stacked transparent U-shaped acrylic risers 15 by 15 by 15 cm (SW Plastics F2191, Riverside, CA), that formed a rectangular cage 15 by 15 by 30 cm (width by depth by height) with two open sides. One open face was covered with white semiopaque no-see-um netting (Skeeta, Bradenton, FL) and the other was fitted with a 30-cm-long sleeve sewn from no-see-um netting.

Approximately 18 cages per week (6 each every Monday, Wednesday, and Friday) were prepared for *D. citri* oviposition. Approximately 15–20 adult *D. citri* were introduced into each cage and allowed to oviposit for 2–4 d, after which they were removed and transferred to a new cage for oviposition. *D. citri* adult

mortality was mitigated by supplementing additional individuals sourced from HLB-free California strain laboratory colonies. Inoculated experimental cages were then placed within a larger secondary outer cage (BugDorm model 6610, MegaView Science, Taichung, Taiwan), and grouped by inoculation date. This dual-cage system was instituted in compliance with designated protocols under CDFA Permit 2870 to ensure *D. citri* adults would not escape from cages.

Colony plants were watered three times per week (Monday, Wednesday, and Friday). All *D. citri* colonies in IQF rearing rooms were maintained under constant conditions at 29°C, 40% RH, and a photoperiod of 14:10 (L:D) h. Cages were illuminated artificially (Sylvania Fluorescent Octron 4100K bulbs, Osram Sylvania Inc., Danvers, MA) and they had exposure to natural daylight from a single south-facing window.

Maintenance of NTP Colonies. Native psyllid species (*Heteropsylla* sp., *H. texana*, *D. fremontiae*, and *B. cockerelli*), as well as *B. melaleucae*, were maintained in colonies on their preferred host plants [*Acacia farnesiana* (L.) Willderman, *Prosopis glandulosa* Torrey, *Fremontodendron californicum* (Torrey) Coville, *Solanum melongena* L., and *M. quinquenervia*, respectively] in IQF rearing rooms. *Arytainilla spartiophylla* (Forester) and *Euphyllura olivina* (Costa), two nonnative species, were not maintained in colonies in IQF because they are not subject to parasitism in the field (Percy et al. 2012) and nymphs were field-collected on *Cytisus scoparius* (L.) Link and *Olea europaea* L., respectively, on an as-needed basis.

To initiate NTP colonies, four to six host plants were placed within a large BugDorm (model 2120, MegaView Science, Taichung, Taiwan) and NTP adults were introduced into cages. Colonies were maintained by rotating out older host plants for new plants with appropriate growth stages for oviposition on an as-needed basis. *A. farnesiana* and *P. glandulosa* were grown from seed and matured in 3.8-liter pots (16 cm in diameter by 18 cm in depth) using modified UCR type III potting mix. *S. melongena* ("Long Purple" variety, Botanical Interests, Broomfield, CO), *F. californicum* (Moosa Creek Nursery, Valley Center, CA), and *M. quinquenervia* (Ponto & Sons Wholesale Nursery, Vista, CA) were obtained as seedlings and transplanted into 3.8-liter pots. Host plants were pruned on an as-needed basis to promote flush growth, as well as to restrict plant height so plants could fit within cages. All plants were grown in UCR AgOps greenhouses and watered every Monday, Wednesday, and Friday. Miracle-Gro all-purpose plant food (water soluble formula, 24-8-16 NPK; The Scotts Miracle-Gro Company, Marysville, OH) was applied as needed. Nontarget host plants were transferred to IQF and placed within the appropriate colony cage when needed. All NTP colonies were maintained in compliance with CDFA Permit Nos. 2976 and 2958 in IQF at 25°C, 40% RH, and a photoperiod of 14:10 (L:D) h.

Plant Preparation for Testing. All NTP were tested on their preferred host plant (Table 1) except *B. cockerelli*, which was kept in colony on *S. melongena* but tested on *Capsicum annum* L. ("California Wonder," Ferry Morse Seed Company, Felton, KY). All host plants used in experiments, with the exception of *C. scoparius*, *F. californicum*, and *M. quinquenervia*, were young seedlings, ~5–7 cm in height, grown from seed in white plastic Ray Leach Cone-tainers (SC7 Stubby, 114 ml, 3.8 cm in diameter, Stuewe and Sons Inc., Portland, OR) in outdoor greenhouses at CDFA Mt. Rubidoux Field Station and delivered as needed to UCR AgOps. *C. scoparius* seedlings were harvested at the site of *A. spartiophylla* collection in El Dorado County, CA, and transported to IQF under CDFA Permit No. 2977, where seedlings were pruned, transplanted into Cone-tainers, and allowed to root before being used.

Seedlings were transported from AgOps greenhouses to an IQF Insectary-level laboratory for preparation at least 2 h before testing commenced. Exposure vials were assembled by taking seedlings in Cone-tainers and stripping them of excess foliage. A 3.7-cm-diameter netted upholstery foam disc was fitted around the base of the seedling stalk to completely cover the soil to prevent soil pest contamination of testing vials, as well as to prevent both psyllid nymphs and *D. aligarhensis* from becoming lost in soil. A ventilated 148-ml transparent plastic vial (Thornton Plastic Co., Salt Lake City, UT) was then affixed to the Cone-tainer to provide containment for insects: a 3.7-cm-diameter hole was cut into the vial lid, which allowed it to be fitted around the top of the Cone-tainer, and was held in place using brass thumbtacks. Vials were ventilated with three 2-cm-diameter holes (two on either side and one on the base) covered with fine organza mesh, inverted, and placed over the top of the seedling and fastened onto its corresponding lid (Fig. 1A). Five second- to third-instar *D. citri* nymphs were transferred with a fine-hair paintbrush from colony plants onto prepared *C. volkameriana* seedlings, and five second- to third-instar nymphs of the NTP being tested were transferred in a similar manner from their respective colony plants onto corresponding host seedlings.

Because *F. californicum* and *M. quinquenervia* were obtained in D40 conical growing containers (6.5 cm in diameter, 25 cm in depth) and were too large to transplant into Cone-tainers, a modified experimental design was developed for testing these two NTP species (Fig. 1B). A system similar to the Cone-tainer setup was adapted using 470-ml clear plastic cups and corresponding 10.5-cm-diameter lids instead of ventilated vials. A 6-cm-diameter hole was cut in the center of each lid, which was then fitted around the top of the D40 cone and held in place with a rubber band. The base of each cup was removed and covered with white no-see-um netting to provide ventilation. A 7-cm paper disc was fitted around the base of the seedling and affixed to the top rim of the D40 to cover the soil in the manner of the foam discs in Cone-tainers. Five each of *D. fremontiae* and *B. melaleucae* nymphs were transferred onto seedlings in these modified D40 growing containers for testing in the same manner as other NTP species. Two small drops of wild clover honey were applied to the inner walls of all testing arenas to provide a carbohydrate source to *D. aligarhensis*.

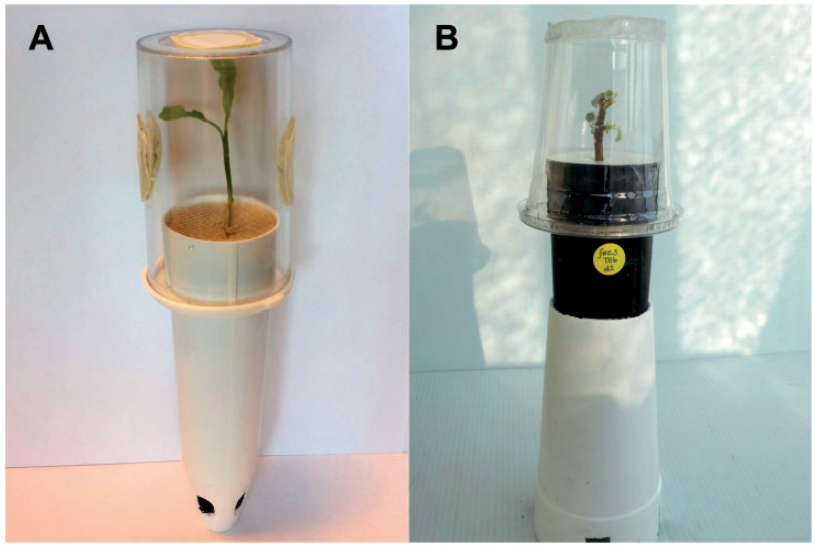

Fig. 1. Experimental setup of seedlings used in host range testing. (A) Cone-tainer setup used for testing *D. citri*, *B. cockerelli*, *Heteropsylla* sp., *A. spartiophylla*, *E. olivina*, and *H. texana*. (B) Modified D40 setup used for testing *D. fremontiae* and *B. melaleucae*.

Experimental Design. To perform exposure trials, one mated female *D. aligarhensis* was introduced into the Cone-tainer or enclosed D40 cone containing psyllid nymphs on their respective host plants. Experiments were set up in a block design composed of eight treatment types per NTP species, so that all treatments were tested simultaneously, and each block of treatments lasted 48 h. Each NTP trial was replicated 8–10 times at the average rate of two replicates per week over a 4-wk period. All experiments took place in a quarantine laboratory at 27°C, 40% RH, and a photoperiod of 14:10 (L:D) h.

A combination of sequential no-choice, static no-choice, and choice tests were used to assess the propensity of *D. aligarhensis* to attack different psyllid species, as well as the impact on *D. citri* and NTP nymph survivorship (Table 2). Control treatments were run for both *D. citri* and NTP in the absence of *D. aligarhensis* to provide baseline measurements of psyllid nymph mortality and survivorship under identical conditions as parasitoid exposure treatments. Following completion of testing, surviving *D. aligarhensis* were immediately preserved in 95% ethanol.

Sequential No-Choice Tests (T1, T2, T5, and T6). Sequential no-choice treatments exposed *D. aligarhensis* to either *D. citri* or NTP for the first testing period, which varied according to either short (i.e., 4 h) or prolonged (24 h) exposures, and subsequently *D. aligarhensis* was transferred onto the opposite host type (i.e., *D. citri* to NTP or NTP to *D. citri*) for the second testing interval of the same exposure period. This sequential setup evaluated whether the order of host exposure and the total length of exposure time were significant factors influencing attack rates.

Short sequential no-choice treatments had female *D. aligarhensis* in vials with either five *D. citri* (T1) or five NTP (T2) nymphs on their respective host plants and allowed to oviposit for 4 h. Females were subsequently removed and directly transferred to a vial with the opposite psyllid type (i.e., onto NTP for T1 and onto *D. citri* for T2) and allowed to oviposit for an additional 4 h. After this second 4-h exposure period, females were removed in 2-ml O-ring microcentrifuge tubes with honey droplets and stored overnight for 16 h at ~14°C. The same exposure sequence was followed on day 2 using the same *D. aligarhensis* female as day 1 (Table 2). In prolonged sequential exposures, female *D. aligarhensis* were introduced into vials containing either 5 *D. citri* (T5) or 5 NTP (T6) nymphs on their respective host plants on day 1 and allowed to oviposit for 24 h. On day 2, females were transferred directly to new vials with the reciprocal host species and given an additional 24 h to oviposit before removal.

Prolonged Static No-Choice Tests (T4 and T7). Static tests exposed *D. aligarhensis* to the same host species over the entire testing cycle. Females were introduced into vials with either five *D. citri* (T4) or five NTP (T7) nymphs for the initial 24 h exposure; they were then directly transferred to a second vial containing the same host species for a second 24-h exposure period. This allowed *D. aligarhensis* time to host feed during the first testing period, and was designed to accommodate an increased likelihood of oviposition on psyllid hosts presented on the second day of testing.

Choice Tests (T3). Choice tests presented both target and nontarget hosts simultaneously in a shared testing arena to assess host preferences when foraging females could choose between host species. Unenclosed *D. citri*-infested *C. volkameriana* seedlings and NTP-infested seedlings were used in choice tests and allowed free access to both species of psyllid by female

Table 2. Summary of treatment types and experimental design of host range trials

Treatment	Exposure type	Psyllid species exposed to *D. aligarhensis*			
		Day 1		Day 2	
		1st 4 h	2nd 4 h	1st 4 h	2nd 4 h
T1	Short sequential[a]	*D. citri*	NTP[b]	*D. citri*	NTP
T2	Short sequential[a]	NTP	*D. citri*	NTP	*D. citri*
		24 h		24 h	
T3	Choice	*D. citri* + NTP		*D. citri* + NTP	
T4	Prolonged static	*D. citri*		*D. citri*	
T5	Prolonged sequential	*D. citri*		NTP	
T6	Prolonged sequential	NTP		*D. citri*	
T7	Prolonged static	NTP		NTP	
T8	Control	*D. citri* + NTP		*D. citri* + NTP	

[a] *D. aligarhensis* in short-term treatments held overnight (16 h) with access to honey droplets at 13.2–14.4°C.
[b] Nontarget psyllid (NTP).

D. aligarhensis. Seedlings were placed within a choice testing arena (a mesh and acrylic cage 15 by 15 by 20 cm for NTP species in Cone-tainers, and 15 by 15 by 30 cm for NTP species in D40s), into which one *D. aligarhensis* female was released. After 24 h, nymphs were removed from the testing arena and replaced with new hosts of the same species to which the same *D. aligarhensis* female was exposed to for an additional 24-h period. At the end of each exposure period, all choice treatment plants were fitted with ventilated vials to contain emerging psyllids and parasitoids.

Control (T8). One vial each of *D. citri* and NTP were prepared in an identical manner as exposure treatments, but nymphs were not exposed to parasitoids. These control treatments measured psyllid survivorship in the absence of *D. aligarhensis* under the same handling procedures and experimental conditions as exposure treatments. Controls were run for both day 1 and day 2 of each replicate.

Data Recording and Statistical Analyses. All test vials were observed at 6–8 d, 12–14 d, and 21 d after initial replicate setup to record psyllid and parasitoid emergence rates. Any remaining nymphs (i.e., no emergence of parasitoids or eclosion of adult psyllids) counted during this third observation were considered dead. Recorded outcomes for psyllid nymphs fell into four categories: live adult (i.e., psyllid nymph successfully eclosed), mummy (i.e., successful parasitism by *D. aligarhensis* resulted in a mummy), dead (i.e., dead psyllid nymph recovered), and missing (i.e., psyllid nymph not recovered). Missing nymphs were not included in data figures because their fate could not be conclusively determined. Parasitized psyllid nymphs (mummies) were monitored for emergence of adult parasitoids, which were counted and sexed.

Operating under the null hypothesis that the rate of psyllid mortality was independent of the presence or absence of parasitoids, Fisher's exact test for count data was run to compare each treatment against the control mortality for both *D. citri* and NTP. Experiment-wide error rate of multiple comparisons was controlled using the sequential Bonferroni correction method, where experimental *P*-values were ranked lowest to highest and compared in a step-down method against a significance level of $0.05/n$, $0.05/n - 1$, $0.05/n - 2$, etc. (where n = the number of comparisons). One species (*B. cockerelli*) of the seven NTP tested was parasitized by *D. aligarhensis* (see Results), so a second Fisher's exact test with sequential Bonferroni correction was run using a pairwise comparison of treatments between *D. citri* and *B. cockerelli* to determine if *D. aligarhensis* exhibited a preference between these two psyllid species. Analyses of psyllid nymph mortality were conducted using basic packages included in the R statistical programming environment (R version 3.0.2, R Core Team 2013, The R Foundation for Statistical Computing, Vienna, Austria).

Results

***D. citri* Mortality for Control and Parasitoid Exposure Treatments.** *D. citri* in control cages experienced an average mortality rate of ~16% across all trials, with ~58% successfully eclosing as adult psyllids. The ~26% discrepancy is attributed to psyllid nymphs which were unaccounted for (i.e., missing) at time of data collection. There was no parasitism recorded in any control treatment, indicating that unintentional exposure of nymphs to *D. aligarhensis* did not occur at any time.

Parasitism of *D. citri* by *D. aligarhensis* occurred in every treatment type and in every replicate. Total *D. citri* nymph mortality (death due to unknown causes + death by parasitism) was significantly elevated across all treatments, regardless of exposure type or length, in most replicates (see Table 3 for full summary of results and statistical analyses). For those sets of *D. citri* exposures that were not significantly elevated (i.e., the *H. texana*, *D. fremontiae*, and *B. melaleucae* exposure experiments), this resulted from high mortality in control treatments from unknown causes. Rates of *D. citri* parasitism by *D. aligarhensis* across all exposure types in all replicates averaged 22%, and ranged from 4 to 50% within individual treatments (Table 3).

Short sequential no-choice trials with *D. citri* exposed first (T1) had an overall mortality rate (death by unknown causes + parasitism) across all replicates of 46% and ranged from 20 to 52%. NTP-first short

Table 3. Mortality and parasitism rates for second- and third-instar *D. citri* and NTP nymphs when exposed to *D. aligarhensis* females under different exposure treatments

Treatment	Total nymphs exposed	Parasitism (%)	Total mortality (%)	*P*-value	Total nymphs exposed	Parasitism (%)	Total mortality (%)	P-value
		D. citri				*B. cockerelli*		
Short sequential *D. citri* first	100	39	52	<0.01[a]	90	13	32	<0.01[a]
Short sequential NTP first	100	40	46	<0.01[a]	95	19	24	<0.01[a]
Choice test	100	27	33	<0.01[a]	95	17	31	<0.01[a]
Prolonged sequential *D. citri* first	50	46	60	<0.01[a]	50	8	36	<0.01[a]
Prolonged sequential NTP first	50	38	54	<0.01[a]	50	10	44	<0.01[a]
Prolonged exposure (static)	95	51	58	<0.01[a]	100	12	35	<0.01[a]
Control (No parasitoid)	105	0	6	–	95	0	4	–
		D. citri				*Heteropsylla* sp.		
Short sequential *D. citri* first	80	28	48	<0.01[a]	80	0	8	0.31
Short sequential NTP first	80	19	44	<0.01[a]	80	0	5	0.71
Choice test	75	21	35	<0.01[a]	75	0	16	<0.01[a]
Prolonged sequential *D. citri* first	40	40	60	<0.01[a]	40	0	5	0.64
Prolonged sequential NTP first	40	25	53	<0.01[a]	35	0	11	0.10
Prolonged exposure (static)	80	31	48	<0.01[a]	80	0	9	0.19
Control (no parasitoid)	85	0	11	–	90	0	3	–
		D. citri				*A. spartiophylla*		
Short sequential *D. citri* first	80	18	34	0.02	80	0	39	0.44
Short sequential NTP first	75	16	33	0.03	75	0	32	1.00
Choice test	80	29	40	<0.01[a]	80	0	13	<0.01[a,b]
Prolonged sequential *D. citri* first	40	20	33	0.07	30	0	17	0.11
Prolonged sequential NTP first	40	3	13	0.61	40	0	23	0.31
Prolonged exposure (static)	70	31	44	<0.01[a]	70	0	30	0.87
Control (no parasitoid)	90	0	18	–	105	0	32	–
		D. citri				*E. olivina*		
Short sequential *D. citri* first	80	21	35	<0.01[a]	75	0	20	0.70
Short sequential NTP first	65	29	49	<0.01[a]	70	0	20	1.00
Choice test	80	18	39	<0.01[a]	80	0	13	0.23
Prolonged sequential *D. citri* first	40	18	33	0.03[a]	35	0	6	0.06
Prolonged sequential NTP first	40	18	23	0.32	40	0	33	0.13
Prolonged exposure (static)	75	41	55	<0.01[a]	70	0	19	0.85
Control (no parasitoid)	100	0	15	–	100	0	20	–
		D. citri				*H. texana*		
Short sequential *D. citri* first	85	20	38	0.08	85	0	24	0.86
Short sequential NTP first	90	20	32	0.33	90	0	13	0.05
Choice test	90	23	46	<0.01[a]	90	0	8	<0.01[a,b]
Prolonged sequential *D. citri* first	45	11	31	0.54	45	0	11	0.08
Prolonged sequential NTP first	45	18	38	0.24	45	0	18	0.40
Prolonged exposure (static)	85	14	28	0.62	85	0	26	1.00
Control (no parasitoid)	100	0	25	–	100	0	25	–
		D. citri				*D. fremontiae*		
Short sequential *D. citri* first	80	8	33	0.18	80	0	11	0.63
Short sequential NTP first	80	23	48	<0.01[a]	80	0	14	0.35
Choice test	75	15	32	0.23	75	0	8	1.00
Prolonged sequential *D. citri* first	40	18	35	0.20	35	0	20	0.12
Prolonged sequential NTP first	25	16	40	0.13	40	0	20	0.09
Prolonged exposure (static)	80	31	50	<0.01[a]	70	0	7	0.78
Control (no parasitoid)	100	0	23	–	100	0	9	–
		D. citri				*B. melaleucae*		
Short sequential *D. citri* first	75	11	20	0.85	75	0	3	1.00
Short sequential NTP first	80	11	16	0.84	80	0	1	0.63
Choice test	70	9	30	0.09	70	0	13	0.01[a]
Prolonged sequential *D. citri* first	40	10	30	0.17	40	0	8	1.00
Prolonged sequential NTP first	35	23	37	0.03[a]	40	0	0	0.26
Prolonged exposure (static)	80	16	33	0.04[a]	70	0	4	0.68
Control (no parasitoid)	100	0	18	–	105	0	3	–

P values generated using Fisher's exact test for count data. Some percentages may not add up to 100% because of rounding.
[a] Fisher's exact test with sequential Bonferroni correction comparing mortality versus control was significant.
[b] Results were significant owing to treatments having significantly lower mortality than control.

sequential no-choice exposures (T2) had an overall *D. citri* mortality rate of 38% with a range of 16–49% across all replicates. A mortality range of 30–45% was observed in choice exposures (T3), with an overall mortality rate across all replicates of 38%. Prolonged static exposures (T4) consistently yielded higher mortality rates than other treatment types in nearly all experiment sets, with an overall rate of 46%, which ranged from 28–58%. The overall mortality rate for prolonged sequential exposures when *D. citri* was exposed first (T5) was 41%, with a range of 30–60%. Prolonged sequential exposures where NTP were exposed first (T6) showed an overall mortality of 37% and a range of 12–54% (Table 3).

Table 4. Comparison of *D. aligarhensis* parasitism and nymph mortality between *D. citri* and *B. cockerelli* across different treatment types

Treatment	Host species	Total nymphs exposed	Parasitism (%)	Total mortality (%)	*P*-value
Sequential *D. citri* first	*D. citri*	100	39	52	<0.01[a]
	B. cockerelli	90	13	32	
Sequential NTP first	*D. citri*	100	40	46	<0.01[a]
	B. cockerelli	95	19	24	
Choice test	*D. citri*	100	27	33	0.76
	B. cockerelli	95	17	31	
Prolonged exposure *D. citri* first	*D. citri*	50	46	60	0.03[a]
	B. cockerelli	50	8	36	
Prolonged exposure NTP first	*D. citri*	50	38	54	0.42
	B. cockerelli	50	10	44	
Prolonged exposure (static)	*D. citri*	95	51	58	<0.01[a]
	B. cockerelli	100	12	35	

P values generated using Fisher's exact test for count data.
[a] Fisher's exact test with sequential Bonferroni correction was significant.

NTP Mortality. Of the seven tested NTP species, *D. aligarhensis* parasitized only *B. cockerelli*, the pestiferous potato psyllid. In total, 67 nymphs of the 480 exposed (14%) were successfully parasitized. Levels of psyllid nymph mortality (death + parasitism) in all treatments were significantly higher than mortality in control treatments (24–44% vs. 4%, respectively; Table 3). However, rates of *D. citri* mortality were always higher when exposed to *D. aligarhensis*, regardless of treatment, than that experienced by *B. cockerelli*. When *B. cockerelli* was examined for host preference through a secondary Fisher's exact test directly comparing mortality between *D. citri* and *B. cockerelli* treatments, rates of NTP mortality were significantly elevated in T1, T2, T4, and T7 treatments (Table 4). It is likely that elevated mortality rates in the prolonged static no-choice exposure trials (i.e., T4 and T7) were, in part, due to prolonged confined exposure, which prevented patch abandonment by *D. aligarhensis*. In prolonged sequential tests where *D. citri* was presented first, there was a significantly higher rate of *D. citri* (60%) versus *B. cockerelli* (36%) mortality, though there was no significant difference when *B. cockerelli* was presented before *D. citri*. An analysis between *D. citri* and *B. cockerelli* mortality rates (27 and 17%, respectively) in choice cages showed no statistical difference (Table 4).

E. olivina, an invasive pest infesting olive trees, was chosen as a taxonomically closely related species to *D. citri* for testing (representing a different tribe within Liviidae; Table 1). Mortality of *E. olivina* was ≤20% in all treatment types except prolonged exposure, where NTP was presented first, which was 33%. No mortality for any treatment type when exposed to *D. aligarhensis* was significantly elevated from the control rate (20%). *D. citri* control mortality was 15% for exposure trials with *E. olivina*, and all treatments exposed to *D. aligarhensis*, with the exception of prolonged sequential no-choice exposures where NTP was presented first, were significantly elevated (Table 3).

The native California psyllid, *D. fremontiae*, was the second species chosen for its phylogenetic proximity to *D. citri* (representing a third tribe within Liviidae; Table 1). Control mortality of *D. fremontiae* was 9%, and there was no significant difference between control and any NTP treatment exposed to *D. aligarhensis* (mortality rates ranged from 7 to 20%). While *D. citri* mortality rates were significantly higher than control (23%) only in short sequential no-choice tests (NTP first) and prolonged static no-choice exposures to *D. aligarhensis*, mortality levels across all *D. citri* exposures was significantly higher (>30%) when compared with *D. fremontiae* (Table 3).

H. texana nymph mortality in control treatments was 25%, and though there were significant differences between levels of NTP control versus *D. aligarhensis* exposure mortality in short sequential no-choice tests with NTP exposed first and choice exposures, this significance was owing to mortality rates in exposure cages being markedly lower than in control cages (13, 8, and 11%, respectively). *D. citri* mortality ranged from 28 (prolonged static no-choice exposures) to 46% (choice trials), though only the choice treatment yielded significantly higher mortality as compared with control cages (25%; Table 3).

Heteropsylla sp. control mortality (3%) was not significantly different from the NTP mortality rates observed in any *D. aligarhensis* exposure treatments, which ranged from 5 (short sequential no-choice exposures presenting NTP first) to 11% (prolonged sequential no-choice exposures presenting NTP first). Conversely, *D. citri* mortality was significantly elevated in every treatment exposed to *D. aligarhensis*, ranging from 35 (choice) to 60% (prolonged sequential presenting *D. citri* first) as compared with an 11% mortality rate in the control treatment (Table 3).

A. spartiophylla, a self-introduced species in northern California, is a fortuitous biological control agent of the invasive noxious weed, Scotch broom, *C. scoparius*. Mortality rates in *A. spartiophylla* control cages (32%) did not differ significantly when compared with treatments exposed to *D. aligarhensis* (which ranged from 23 to 39%), except in choice cages, where NTP nymph mortality was significantly lower than the controls (Table 3). *A. spartiophylla* nymphs likely suffered elevated mortality because of the nature of their field

collection, excessive handling, and transportation to IQF before testing. *D. citri* mortality was significantly higher in all treatments exposed to *D. aligarhensis* than in control cages (33–44% vs. 18%), except in prolonged sequential no-choice trials (13 and 33%; Table 3).

B. melaleucae mortality across all exposure treatments was low (0–13%), and only the mortality rates observed from choice trials (13%) were significantly higher than the observed <3% control mortality (Table 3). It is unknown what may have contributed to this elevated mortality rate in choice cages, but no parasitism was observed. Associated *D. citri* treatments experienced a lower rate of parasitism and combined mortality, with only the prolonged sequential no-choice exposures being significantly higher than controls (37 and 33% vs. 18%; all other exposures were <30%; Table 3).

Discussion

D. aligarhensis is reportedly highly host specific, with records indicating *D. citri* as its only known host (Aubert and Quilici 1984, Skelley and Hoy 2004). Host range trials for *D. aligarhensis* conducted here were comprised seven NTP species spanning four families and seven tribes, including four species native to California, one invasive pest species, and two nonnative species used for biological control of invasive weeds. Results from no-choice and choice trials presented here largely support this observation, as only one nontarget species, the highly pestiferous potato psyllid, *B. cockerelli*, was successfully parasitized.

Under a combination of short sequential, prolonged sequential, prolonged static no-choice, and choice exposure trials, *D. aligarhensis* was shown to successfully parasitize only *D. citri* (target host) and *B. cockerelli* (nontarget). Compared with the attack rates on *D. citri* (~40% parasitism), *B. cockerelli* experienced an overall average parasitism rate of 14% by *D. aligarhensis*. Because these two psyllid species are not closely related phylogenetically, it is likely that observed parasitism was the result of artificial testing conditions or a similarity in size and superficial morphology to *D. citri* nymphs. A similar result with *B. cockerelli* was observed in *T. radiata* host range testing (Hoddle and Pandey 2014), but parasitism of *B. cockerelli* by *T. radiata* in the field has not been observed even in areas of very close sympatry (i.e., infested host plants growing together in gardens; M. S. H, unpublished data). These low levels of nontarget parasitism can often be the result of small-scale testing schemes, which artificially inflate the proportion of nontarget species estimated to be at risk of attack in the field (van Lenteren et al. 2006). There is also a possibility that host volatiles, which many parasitoids used for recognition of suitable hosts (Zuk and Kolluru 1998), are similar between the two species, leading *B. cockerelli* to be mistakenly identified by *D. aligarhensis* as a suitable host in small cage trials. However, additional research into chemical cues used by various psyllid species and associated parasitoids is needed to determine if this is the case (Arras et al. 2012). Unlike *B. cockerelli*, *E. olivina*, and *D. fremontiae*, the two NTP most closely related taxonomically to *D. citri* experienced no parasitism. This result may support the suggestion that *D. aligarhensis* nontarget host selection relies more on size or morphology similarity of nymphs than species relatedness to the target, *D. citri*.

Results presented here suggest that potential for *D. aligarhensis* to inflict significant nontarget impacts in nature will likely be negligible, and attacks on *B. cockerelli*, should they occur in the field, are unlikely to affect populations of this pest. In addition, *D. aligarhensis* will be faced with competition from a rich guild of native parasitoids that attack native California psyllids (Percy et al. 2012). This type of biotic resistance may be important for minimizing population-level nontarget impacts by parasitoids released for *D. citri* biological control (Hoddle and Pandey 2014). Data from this research indicate that *D. aligarhensis* is very unlikely to have deleterious effects on *A. spartiophylla* or *B. melaleucae*, two important psyllid species attacking either Scotch broom or melaleuca, invasive weeds found in northern California and the Florida Everglades, respectively.

The ability of *D. aligarhensis* to establish populations in California is of high interest. Though this parasitoid has been continuously released in Florida since 1998, there remains no conclusive evidence that it has established (Rohrig et al. 2011). Unlike the strains released in Florida, which were all-female and confirmed to be infected with *Wolbachia* (which can influence survivorship, fecundity, and offspring sex ratio; Skelley and Hoy 2004, Rohrig et al. 2012), the population of *D. aligarhensis* used in these studies is bi-parental. It is also worth noting that, while the majority of *D. aligarhensis* releases in Florida took place in commercial citrus groves, which may have been subjected to pesticide treatments, the majority of California releases of *D. aligarhensis* are expected to occur primarily in residential areas, where pesticide use is very low and *D. citri*-infested citrus is common.

Possible competition with *T. radiata* is another concern in establishing *D. aligarhensis* as a biological control agent of *D. citri*. *T. radiata* has a higher reproductive rate and shorter generation time (nearly two generations per one generation of *D. aligarhensis*), which may allow *T. radiata* to competitively exclude *D. aligarhensis* in areas where they could be competing for *D. citri* nymphs. In addition, *T. radiata* females have been recorded to kill nearly twice as many *D. citri* nymphs in their lifetime as *D. aligarhensis* through a combination of parasitism and host feeding (Skelley and Hoy 2004). In cases of direct competition, *T. radiata* has been demonstrated to successfully parasitize *D. citri* nymphs within 5 d following initial oviposition by *D. aligarhensis* (Rohrig et al. 2012). The advantage posed by the situation in southern California is that, although *T. radiata* has small established populations as a result of the biological control program targeting *D. citri* (Hoddle and Pandey 2014), there remain large areas where *D. citri* is present that have no established *T. radiata* populations. Selection of these areas (e.g., San Diego and Imperial Counties; see

distribution map in Morgan et al. 2014) for *D. aligarhensis* releases may increase establishment rates because of reduced interspecific competition for *D. citri* nymphs. In addition, varied climatic conditions throughout major citrus production areas in California may provide climate niches more favorable to either *D. aligarhensis* or *T. radiata*. Some notable past biological control successes of citrus pests in California have required more than one natural enemy because of differential performances by biological control agents in citrus production areas with different climates (Quezada and DeBach 1973). However, there is debate over the number of natural enemy species that need to be established to achieve successful insect pest suppression (Denoth et al. 2002).

It is possible that California could provide a permissive environment for proliferation by *D. aligarhensis* because the new range into which this parasitoid may be introduced could lack hyperparasitoids. In regions where *D. aligarhensis* is native, it is known to be attacked by at least 10 species of hyperparasitoids, which resulted in ~40% hyperparasitism in Taiwan, compared with <1% of *T. radiata* being hyperparasitized (Chien et al. 1989). In Pakistan, from where the California populations of *D. aligarhensis* were sourced, it is also attacked by several species of hyperparasitoid (Hoddle et al. 2013, Bistline-East and Hoddle 2014). Consequently, introduction into novel areas free of coevolved hyperparasitoids may facilitate *D. aligarhensis* establishment and allow the development of higher populations in California than those observed in its native range (Simmons et al. 2013).

A goal of the classical biological control program in California is to reconstitute the guild of primary *D. citri* parasitoids from Punjab, Pakistan, an area with a very good climate match to citrus production areas in California (Hoddle 2012). Should *D. aligarhensis* and *T. radiata* both establish in southern California, greater control of *D. citri* populations may be expected, owing to complementarity, than would be possible with either species of parasitoid individually. Use of biological control to suppress *D. citri* populations, especially in areas where pest populations are high but insecticide treatments are unlikely for area-wide suppression of pest management, may reduce the spread of HLB from urban zones (HLB was first detected in a backyard tree in California; Kumagai et al., 2013) to commercial production areas as vector prevalence is minimized (Pelz-Stelinski et al., 2010). This reduction of vector densities in residential areas to reduce the rate of HLB spread into commercial citrus production zones is a key goal of California's *D. citri* biological control program. On 1 November 2013, an 86-page Environmental Assessment Report detailing the research reported here was submitted to USDA-APHIS. On 26 October 2014, following review of this report, USDA-APHIS issued a finding of no significant impact for *D. aligarhensis*, and on 24 November 2014, USDA-APHIS issued permit P526P-14-04034, authorizing the release of *D. aligarhensis* from quarantine, allowing releases in California to begin in December 2014.

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