Research Project Progress Report

Classical biological control of Asian citrus psyllid with *Tamarixia radiata* in urban Southern California

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A biological control program for Asian citrus psyllid (ACP), *Diaphorina citri*, in Southern California was initiated in September 2010 when the authors visited the University of Agriculture in Faisalabad Pakistan to assess the feasibility of using this campus as a home base for foreign exploration efforts to find and collect ACP natural enemies (see *Citrograph* Sept/Oct. 2010, page 30-33 for more on this initial trip).

In the intervening two years, a lot has been accomplished with cooperators in Pakistan (especially Vice Chancellor Iqrar Kahan [a UCR alumnus] and Dr. Mohammad Jalal Arif), colleagues at UC Riverside (in particular the Stouthamer Lab), with the citrus industry, the California Department of Food and Agriculture, USDA, and homeowners with ACP-infested citrus in Los Angeles, Orange, Riverside, and San Bernardino Counties.

***Tamarixia radiata* is a parasitoid that attacks fourth and fifth instar Asian citrus psyllid nymphs. This parasitoid from Pakistan is being released for the classical biological control of Asian citrus psyllid in California.** *Photo by Mike Lewis, Center for Invasive Species Research, UC Riverside.*

This article provides the latest updates on the ACP biocontrol program in California with *Tamarixia radiata* (Hymenoptera: Eulophidae) a host-specific parasitoid imported into Southern California from the Pakistan Punjab. Punjab was selected for natural enemy collections for two major reasons: (1) it has a climate similar to California, and (2) it is part of the area of origin for ACP.

The prevailing climate in the Pakistan Punjab has about a 70% match with major citrus production areas in California. Biological control theory states that natural enemies pre-adapted to the climate of the area into which they are to be introduced should perform better than strains or biotypes of the same species that come from areas with a poor climate match. We considered a 70% climate match between California and the Pakistan Punjab to be very good for this project.

Pakistan is thought to be part of the evolutionary center of origin for ACP. If this assumption is correct, biological control theory suggests that biocontrol agent diversity would be highest here because natural enemies attacking ACP have had the longest time to evolve and diversify into new species on this pest. As you move away from the pest's evolutionary center of origin, towards the margins of its natural range, it is generally thought that natural enemy diversity declines as a result.

So the Punjab of Pakistan, if it is the center of the area of origin for ACP, could offer the greatest chance of finding several different species of natural enemies for use in a classical biological control program in California.

Working with Pakistani researchers

The first major collecting trip to Pakistan for ACP natural enemies was 10 March to 11 April 2011. During this time several major tasks were completed. First, we recruited and trained a Master of Science (MS) student, Mr. Shouket Zaman Khan, under the supervision of Dr. Mohammad Jalal Arif, from the Department of Agri-Entomology at the University of Agriculture (UAF) in Faisalabad.

Zaman's MS research has focused on medium-term population monitoring of ACP (weekly surveys over a 2-year period), parasitism rates, and natural enemy diversity on two types of citrus, Kinnow mandarin and sweet orange at two different research sites at UAF (Square 9 and the Postgraduate Agricultural Research Site [PARS]) (Figure 1).

He has also been responsible for clearing a malaise trap (Figure 2) each week that has been set up in citrus at Square 9. These malaise trap collections provide important information on insect biodiversity associated with citrus in Pakistan, which could reveal new pest and natural enemy species that we are currently unaware of.

Zaman has surveyed other native Pakistani species of *Diaphorina* (the genus to which ACP belongs) to determine how diverse the parasitoid fauna associated with these native psyllids is and whether or not there is overlap in parasitoid species attacking different *Diaphorina* species in the Punjab (Figure 3).

Zaman has also been instrumental in preparing the groundwork, taking care of logistics, and proactively scouting collection sites in advance of collecting trips by the authors. The value of this work cannot be overstated as Zaman's efforts have saved a lot of time and greatly increased the success of collecting trips.

As part of this UCR-UAF collaborative program on ACP, Zaman completed a four-week training period in the Hoddle Lab in September-October 2012. This visit allowed him to be trained in all aspects of the ACP biological control program operating at UCR. Needless to say, it was very exciting for Zaman to work with parasitoids sourced from our collecting trips to Pakistan both in the lab at UCR and at various field sites in Southern California. He also made many new friends during his visit, which undoubtedly was a great thing for building positive Pakistan-USA ties.

Second, during the March-April 2011 visit, Mark Hoddle (MH) and Christina Hoddle (CH) scouted citrus production areas in Sargodha, Gujranwala, Toba Tek Singh, and Faisalabad (Figure 4) with collaborators from UAF who had strong connections to the citrus producing community in these major Punjabi production areas.

A basic field day would consist of an early start (often around 5:30 a.m.), scouting fields in search of ACP-infested trees, and collecting ACP-infested stems to return to the lab for processing that night (Figure 5). Lab work was challenging, especially at night, because load shedding (a euphemism for Pakistan's unpredictable electricity supplies; we were getting an erratic six-hour supply a day) would knock out lights and fans and this meant working with headlamps and flashlights (Figure 6), and sometimes temperatures in the lab would surpass 125°F – this makes you sweat a lot!

Once processed, stems from collection sites were isolated in bugdorms that were labeled by locality and collection date, and parasitoids that emerged were kept separate according to the bugdorm into which they emerged.

Fig. 1. Shouket Zaman Khan and Christina Hoddle conducting weekly Asian citrus psyllid and parasitism surveys at the Postgraduate Agricultural Research Site (PARS), University of Agriculture Faisalabad, Punjab Pakistan.

Fig. 2. Saif-ur-Rehman (right), Christina Hoddle, and Shouket Zaman Khan with the malaise trap set up in a block of Kinnow at Square 9, University of Agriculture, Faisalabad Pakistan. The first Kinnow trees were donated to Pakistan in 1940 by the University of California Riverside. This mandarin was developed by H.B. Frost and released for commercial cultivation by the Citrus Experiment Station in 1935. Kinnow accounts for 85% of citrus production in Pakistan, and fruit are exported internationally.

Fig. 3. Christina Hoddle and Shouket Zaman Khan collecting *Diaphorina aegyptiaca* nymphs from *Cordia myxa* in a cemetery in Faisalabad Pakistan to rear parasitoids to determine if Asian citrus psyllid parasitoids attack other species of *Diaphorina* in Pakistan.

Fig. 4. A map of Pakistan indicating the major citrus growing areas (Sargodha, Toba Tek Singh, Gujranwala, and Faisalabad) that were scouted for Asian citrus psyllid natural enemies. The major point of entry into the Pakistan Punjab is the Allama Iqbal International Airport in Lahore.

Consequently, parasitoids were not mixed across localities or collection dates, and they were hand-carried under USDA-APHIS permits in carefully labeled vials back to the Insectary and Quarantine Facility at UCR. Strict adherence to this collection protocol enabled the establishment of isocage lines of *Tamarixia* on ACP in Quarantine at UCR.

Preserving genetic diversity

Because each parasitoid isocage line represented a unique collection site and date, parasitoids from isocages were never mixed in Quarantine. The idea (promoted by Richard Stouthamer, professor of Entomology at UCR) behind these isocage lines was to preserve as much genetic diversity as possible by inbreeding parasitoid populations in each cage.

This isocage line approach differed significantly from what has been done traditionally, where all parasitoids collected from different locations and times are commingled and allowed to mix freely and cross breed inside mass production cages.

Studies with *Drosophila* (i.e., vinegar flies routinely used in genetics studies) suggested that more genetic variation is preserved with isocage lines because it prevents random matings. As a result of continuous mass random matings, genetic variation decreases as populations uniformly become more and more adapted to prevailing Quarantine conditions.

Fig. 5. Christina Hoddle collecting citrus twigs infested with parasitized Asian citrus psyllid nymphs in Sargodha Punjab Pakistan. Cuttings were returned to the lab for processing.

Although some genetic variation is lost with isoscage lines, it is more easily increased again when inbred isocage lines are allowed to cross with each other in a "mongrel" breeding cage. Reconstitution of genetic diversity is achieved via hybrid offspring production that results from crosses by adults from different isocages. It is these genetically diverse "mongrel" or "hybrid" offspring that are released from Quarantine for the biological control of ACP (see Figure 7).

After the March-April 2011 trip, three more trips to collect parasitoids from Pakistan were made; 4-13 June 2011, 28 October – 4 November 2011, 2-5 June 2012 (see Table 1 for a summary of the ACP parasitoids collected in Pakistan).

Fig. 6. Mark Hoddle processing parasitized Asian citrus psyllid nymphs inside Bugdorms in the laboratory at the University of Agriculture Faisalabad (A) and then the electricity goes out, headlamps are turned on, and the temperature goes up … fast! (B).

These collecting trips and Zaman's MS research were supported, in part, by funds issued by the California Department of Food and Agriculture (CDFA), via the Citrus Health Response Program (CHRP). After each of these collection trips, new isocage lines were established in Quarantine at UCR, and a total of 17 lines resulted.

In addition to *Tamarixia*, other parasitoid species attacking ACP were collected, and these included *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae), and *Psyllaphycus diaphorinae* (Hymenoptera: Encyrtidae). Four isocage lines of *D. aligarhensis* were established in Quarantine, but *P. diaphorinae* colonies were not set up because this parasitoid was collected too infrequently to be considered an important ACP parasitoid.

Two species of hyperparasitoid, that is parasitoids parasitizing ACP parasitoids, were reared out in Quarantine, these being *Marietta leopardina* (Hymenoptera: Aphelinidae) and *Aprostocetus* (*Aprostocetus*) sp. (Hymenoptera: Eulophidae) (Table 1). Careful studies by CH in Quarantine conclusively demonstrated that these two hymenopteran species were not ACP parasitoids, and they readily attacked and emerged from ACP mummies that were parasitized by *Tamarixia* or *D. aligarhensis*. Consequently, all *Marietta* and *Aprostocetus* were killed off in Quarantine once their reproductive biology was understood and it was clear that they were hyperparasitoids.

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Thrips
Leafminer
Katydids
Peelminer
Asian citrus psyllid

Starting in March 2010, Dr. Raju Pandey (a post-graduate researcher in the Hoddle Lab) started safety evaluations for the Pakistani *Tamarixia* (these parasitoids were sourced from the Department of Primary Industries in Florida for these studies and ***none*** of them have been released in California), and this work was supported by the CDFA's Specialty Crops Program and the Citrus Research Board.

Stringent, mandatory safety tests

These safety tests were mandatory and required for review by USDA-APHIS. The issuance of a permit by USDA-APHIS to release *Tamarixia* from Quarantine would only occur if it could be demonstrated that this natural enemy posed no undue risk to California's environment. After about 18 months of work in Quarantine, the safety testing for *Tamarixia* was completed. This allowed the preparation of a 60-page Environment Assessment Report (EAR) for USDA-APHIS to review.

The EAR provided the rationale for a classical biological control program against ACP in California using *Tamarixia*, the selection criteria for seven test species of psyllid (see Table 2 for psyllid species tested and their selection criteria) to determine their suitability as hosts for *Tamarixia*.

A major component of the test psyllid selection process was made possible because of work funded by the Citrus Research Board to develop a checklist of native and introduced psyllid species in California, an area of research that had been neglected for over 20 years (see Percy et al., 2012 Zootaxa 3193: 1-27 for this checklist).

The EAR also covered the design and execution of three different experiments (sequential no choice, static choice and no choice experiments) to determine the host range of *T. radiata* when exposed to the seven test psyllids, and analyses

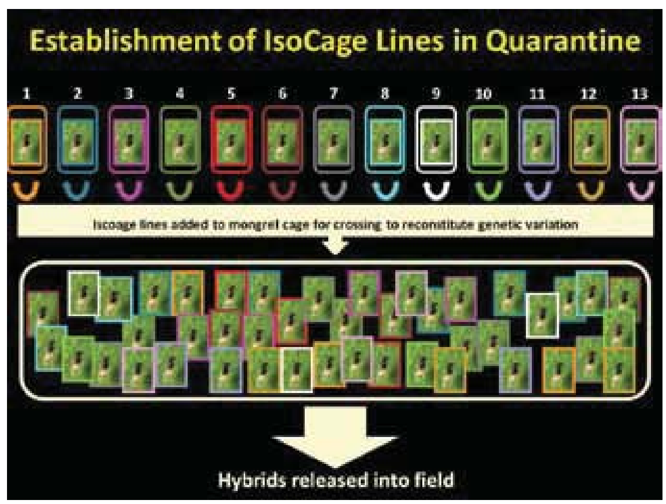

Fig.7. This schematic shows 13 of the 17 isocage lines of Pakistani *Tamarixia radiata* set up in Quarantine at UC Riverside. Offspring produced in each isocage are recycled back into the isocage from which they were derived. Genetic theory suggests that maintenance of these isocage lines helps preserve greater levels of genetic diversity. Diversity is reconstituted when isocage lines are introduced into a mass sting cage where individuals from different lines can freely interbreed. The "mongrel" offspring are expected to have greater genetic diversity because isocage "hybrids" result from random matings. It is these hybrid Pakistani parasitoids with increased genetic diversity that are released against Asian citrus psyllid in California.

of the results of exposure experiments to ascertain the risk *Tamarixa* posed to non-target psyllid species in California.

The results of this painstaking and difficult work clearly demonstrated that *Tamarixia* posed no undue risk to native California psyllids, and it was concluded that this parasitoid was safe to release and establish for the biological control of ACP.

Table 1. Summary of parasitoids (*Tamarixia*, *Diaphorencyrtus*, and *Psyllaphycus*) and hyperparasitoids (*Marietta* and *Aprostocetus*) reared from Asian citrus psyllid nymphs collected from four different trips to the Pakistan Punjab.

Collection Date	No. of *Tamarixia* collected	No. of *Diaphorencyrtus* collected	No. of *Psyllaphycus* collected	No. of *Marietta* collected	No. of *Aprostocetus* collected
10 March – 11 April 2011	80	70	5	0	0
4-13 June 2011	406	25	0	0	0
28 Oct. – 4 Nov. 2011	1012	20	22	52	4
2-5 June 2012	238	164	1	24	3
Grand Total	1736	279	28	76	7

The EAR was submitted to USDA-APHIS on 15 November 2011, and USDA-APHIS issued the release permit (P526P-11-04159) for *Tamarixia* on 7 December 2011. The first release of *Tamarixia* occurred on 20 December 2011 at the citrus Biocontrol Grove at UCR (see *Citrograph* Jan/Feb. 2012, page 11 for more on this initial release).

This was not the first time *Tamarixia* had been released in the USA; it was first deliberately established in Florida for the biological control of ACP in 1999, and the parasitoid is self-introduced into Texas and Mexico.

Extensive survey work in Southern California, especially urban citrus in Los Angeles County, indicated that ACP nymphs were not parasitized and that it was likely that this pest was benefiting, to some degree, from natural enemy-free space.

Establishment of Pakistani *Tamarixia* in heavily infested areas would exert much needed "top-down" pressure on ACP which has the potential to not only reduce pest population densities but to also lower the probability of ACP finding citrus infected with huanglongbing (HLB) (see *Citrograph* May/June 2012 pages 8-9 for more on the HLB find in Los Angeles County.)

Since the initial release of 281 *Tamarixia* into the Biocontrol Grove at UCR, a total of 39,934 (30,788 females and 9,146 males) Pakistani *Tamarixia* reared at UCR have been released at 210 sites across 46 cities in urban areas in Los Angeles, Orange, Riverside, and San Bernardino Counties (see Table 3 for more release details.)

These parasitoids have been produced from two Citrus Research Board projects that are supporting the mass production of *Tamarixia* at UCR (Dr. Anna Soper and Lisa Forster in the Stouthamer lab) and monitoring the impact and spread of *Tamarixia* in urban areas (Ruth Amrich and Allison Bistline in Hoddle lab and Grace Radabaugh with the CDFA).

Confirmed parasitoid activity

Of the 210 sites that have received *Tamarixia*, at least 49 (~23%) of them have evidence of parasitoid activity, mean-

Table 2. The five selection criteria and selected species of non-target psyllids used for host specificity testing of *Tamarixia radiata* in quarantine at the University of California Riverside (UCR). These tests were conducted to determine how broad a host range *T. radiata* would have in California. Ideally a safe and effective natural enemy has a very narrow host range, and therefore poses little undue threat to non-target species, especially native and beneficial psyllids.

Selection criteria	Selected species	Source of test psyllids
Target pest species	*Diaphorina citri* Kuwayama (Liviidae: Euphyllurinae)	USDA-APHIS-PPQ-CPHST Mission Laboratory, Edinburg, TX (certified HLB-free)
1) Close phylogenetic relatedness to D. citri	*Diclidophlebia fremontiae* (Klyver) (Liviidae: Liviinae) (native)	Wrightwood, San Bernardino Co., CA
	Euphyllura olivina (Costa) (Liviidae: Euphyllurinae) (self-introduced pest)	Temecula, Riverside Co., CA
2) Close native host plant relatedness to citrus (Rutaceae: Sapindales)	*Calophya californica* Schwarz (Calophyidae: Calophyinae) (native)	UCR botanical garden, Riverside Co., CA
3) High probability of occurrence in native vegetation surrounding citrus groves	*Heteropsylla texana* Crawford (Psyllidae: Ciriacreminae) (native)	UCR botanical garden, Riverside Co., CA
	Heteropsylla sp. (Psyllidae: Ciriacreminae) (native)	UCR botanical garden, Riverside Co., CA
4) Common native pest psyllid	*Bactericera cockerelli* (Šulc) (Triozidae: no sub-family classification is available for Triozidae) (native)	Trumble Laboratory, Dept. of Entomology, UCR
5) Beneficial psyllid attacking a noxious weed, and functioning as a weed biocontrol agent	*Arytainilla spartiophylla* (Foerster) (Psyllidae: Psyllinae) (self-introduced exotic infesting broom, *Cytisus scoparius*)	El Dorado Co., CA

ing we have recovered ACP nymphs with *Tamarixia* inside them ***and*** we have found ACP nymphs with exit holes from which adult *Tamarixia* have emerged.

This is almost certainly an underestimate of the number of sites *Tamarixia* is likely to have established at, as many release sites have not had repeat visits to assess *Tamarixia* establishment since the initial release. Work this summer is planned to rectify this.

Additionally, following inspection of neighboring properties around these 49 confirmed sites, we have found an additional 19 properties with *Tamarixia* activity, indicating that the parasitoids are moving into neighboring properties without our assistance (again this is likely an underestimate because follow up studies have not yet been made).

In fact, some recoveries have been made up to 7.5 miles from release sites which could indicate that this tiny parasitoid has the ability to spread quickly and over long distances without human assistance (this was seen in the Caribbean and South America).

Dr. Paul Rugman-Jones (Stouthamer lab) has conducted DNA analyses on the parasitoids we have found in the field, and his results provide two very important pieces of information: (1) the DNA confirms that the parasitoids we are finding are of Pakistani origin because their DNA is similar to that analyzed from the colonies in Quarantine at UCR, and (2) there are fairly high levels of genetic diversity in the recovered parasitoids which justifies the efforts that have gone into maintaining isocage lines in Quarantine.

Initial stages 'very encouraging'

The initial stages of this ACP biocontrol program are very encouraging. *Tamarixia* has been cleared from Quarantine and released in Southern California, and it appears to be establishing and spreading on its own. This is remarkable given that relatively few parasitoids have been released so far (< 40,000), releases were made at just over 200 sites, and recoveries have been made at sites where releases were made during the winter, a less than optimal time of year for establishing natural enemies.

The next big steps for the urban monitoring project are to assess the impact *Tamarixia* is having on ACP and what role ants are playing in parasitoid establishment and parasitism rates.

There is also a lot interest in ramping up the production of *Tamarixia*. The USDA is currently working with the Citrus Research Board on the feasibility of this objective, and private insectaries also want to be included in mass production efforts.

Host safety testing for *D. aligarhensis* is currently underway at UCR, and it is anticipated that by Fall 2013 this parasitoid will be cleared for release in California for the biological control of ACP.

Finally, one more collecting trip to Pakistan is being made in April 2013. These collections will add additional *Tamarixia* and *D. aligarhensis* isocage lines in Quarantine, and this new genetic stock could further help to bolster the genetic diversity of these parasitoid populations in California.

An Extension Specialist in biological control, Dr. Mark Hoddle is the director of the Center for Invasive Species Research, University of California Riverside. Christina Hoddle is an Assistant Specialist in the Department of Entomology, UC Riverside.

CRB research project reference number 5500-194.

Table 3. Summary of *Tamarixia* releases in southern California for the classical biological control of Asian citrus psyllid over the period 20 December 2011 to 14 March 2013.

Cities	Counties	No. sites *Tamarixia* released at	No. sites with *Tamarixia* establishment	No. sites with confirmed spread	Total females released	Total males released
Azusa	Los Angeles	8	7	4	1889	716
Beaumont	Riverside	4	0	0	705	140
Bellflower	Los Angeles	3	0	0	439	130
Bell Gardens	Los Angeles	11	9	3	2497	928
Cabazon	Riverside	1	0	0	136	40
Calimesa	Riverside	3	1	0	643	203
Chino	San Bernardino	7	4	0	1672	478
Colton	San Bernardino	1	0	0	145	32
Compton	Los Angeles	5	0	0	874	164
Covina	Los Angeles	1	0	1	23	8
Downey	Los Angeles	10	0	0	1093	280
Duarte	Los Angeles	1	0	0	99	28
Fontana	San Bernardino	9	4	3	1641	550
Fullerton	Orange	4	0	0	662	249
Hemet	Riverside	4	0	0	680	105
Irvine	Orange	2	0	0	295	40
La Habra Heights	Los Angeles	3	0	0	488	92
La Mirada	Los Angeles	3	0	0	459	68
La Puente	Los Angeles	12	4	0	981	251
Long Beach	Los Angeles	6	0	0	424	117
Los Angeles	Los Angeles	17	4	0	3327	1229
Lynwood	Los Angeles	1	0	0	171	44
Menifee	Riverside	1	0	0	234	101
Mira Loma	Riverside	1	0	0	90	18
Montclair	San Bernardino	1	0	0	194	55
Moreno Valley	Riverside	8			1311	245
Norwalk	Los Angeles	3			267	84
Ontario	San Bernardino	3	0	0	288	81
Pico Rivera	Los Angeles	9	4	8	496	217
Pomona	Los Angeles	9	5	0	750	284