Detection of Citrus Huanglongbing Associated ‘Candidatus Liberibacter asiaticus’

in Citrus and *Diaphorina citri*

in Pakistan, Seasonal Variability and Implications on Disease Management

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ABSTRACT

We report the detection of the HLB associated bacterium, ‘*Candidatus* Liberibacter asiaticus’ (Las) from both plants and insects in Pakistan and the seasonal variability in the numbers of Las positive psyllid vector, *Diaphorina citri*. Our studies showed that Las was detectable from trees in areas with maximum temperatures reaching nearly 50°C (average maximum 42°C). However, the bacterium was present at very low levels in psyllids both in summer (June-August) and autumn (September-November) in contrast to reports from Florida, USA, where the bacterium was detectable at very high levels during October-November. We hypothesize that hot summer temperatures in Pakistan may interfere with acquisition and replication of Las in psyllids and may lead to dead and/or non-transmissible Las in plants. Psyllid counts were very low in both summer and winter, showed a population peak (Las positive vectors) in spring, and a larger peak (Las-free psyllids) in autumn. Natural thermotherapy during hot summers and low vector population during environmental extremes may have played a major role in long term survival of the citrus industry in Pakistan. These results may be useful in developing management strategies for US citrus industries in Texas and California.

Additional keywords: Thermotherapy, seasonal variation.
Huanglongbing (HLB) is the most destructive disease of citrus worldwide (12) and is known to be associated with three putative species of phloem-limited α-Proteobacteria, ‘Candidatus Liberibacter asiaticus’ (Las), ‘Ca. L. africanus’ (Laf), and ‘Ca. L. americanus’ (Lam) (39,54) of which Las is prevalent in Asia and the Americas. The disease is transmitted mainly by two psyllid species, Diaphorina citri Kuwayama (Hemiptera: Sternorryncha: Liviidae) and Trioza erytreae (del Guercio) (Hemiptera: Sternorryncha: Triozidae); D. citri is the main vector in Asia and the Americas (14,21,27,29,49). Recently, two additional psyllid vectors, Cacopsylla citrisuga (Hemiptera: Psyllideae) and Diaphorina communis Mathur (Hemiptera: Sternorryncha: Liviidae) have been reported as vectors for Las (15, 22). Although the HLB-associated liberibacters have not been cultured in pure form and Koch’s postulates have not been fulfilled, repeated association of liberibacters with diseased plants has led to the assumption that liberibacters are causal agents of HLB in citrus (12,16).

Citrus is an important fruit crop in Pakistan, cultivated in about 200 thousand hectares with production estimated to be about 2.3 million metric tons (6). Pakistan is ranked 13th in the world in citrus production with about 96% of the total citrus production in the Punjab province (6). Approximately 80% of the cultivated citrus is ‘Kinnow’ mandarin (Citrus reticulata Blanco), grown mainly in Sargodha and its surrounding areas including the districts of Toba Tek Singh, Faisalabad and Sahiwal (41). ‘Kinnow’ and ‘Fewtrell’s early’ mandarin were imported to the Indian subcontinent in 1940 from California and Australia, respectively, and acclimatized well in Punjab (5). Rough lemon (C. × taitensis Risso (= × jambhiri Lush.)) and sour orange (C. × aurantium L.) are the most common rootstocks. The average yield of citrus fruit in Pakistan is low, about 12 tons per hectare which is about 40% of the yield reported in USA (6).
Citrus dieback was a major problem in the Indian subcontinent (includes India, Pakistan and others situated on the Indian plate) dating back to the 18th century (7,13). During the early 1900s, the disease, referred to as citrus decline in the Indian subcontinent was attributed to several biotic and abiotic factors. In 1909, *D. citri* (syn. *Euphalerus citri* (Kuwayama)) was reported to be present on citrus in India in considerable numbers (19,20). Husain and Nath describe dieback and losses associated with *D. citri* infestations in orchards in Sargodha area in 1916 (38). Leaf mottling symptoms that are typical of HLB were also described from this region by Asana (7). A clear association between the psyllid and the disease was demonstrated by Capoor et al., by psyllid transmission in India (14). The suspected presence of HLB in Pakistan and in neighboring India has been documented in several publications (1,2,3,7, 25,38), based mainly on visual symptoms. Although HLB has been known in Pakistan for many decades, adequate information is not available regarding the population dynamics of *D. citri*, appropriate times for effective psyllid control, or optimal time frame for testing psyllids/plants for the presence of HLB-associated bacteria. Chohan et al. demonstrated the presence of HLB in citrus from the North-West Frontier Province of Pakistan (Khyber Pakhtunkhwa) by PCR and sequencing of the *rplKAJL-rpoBC* region of the Las genome (17). To our knowledge, this is the only report of molecular evidence of HLB-associated liberibacters in Pakistan.

HLB has been a challenging disease from both crop management and research perspectives because of various factors including the fastidious nature of the HLB-associated bacteria, irregular distribution, long latency in plants, seasonality, and lower incidence of the bacteria in psyllids (26, 47). In areas where the vectors are present but HLB has not been found, even the most sensitive diagnostic tests available are considered inadequate to certify the area as HLB-free (47). Once the disease is prevalent, management of HLB and maintenance of
productive citrus groves is challenging. Good sources of genetic resistance to HLB in the genus *Citrus* or its relatives have not been identified (4,27). Current attempts to manage HLB, practiced in most of the citrus growing regions of the world, involve continuous prevention of introduction on new plants by use of pathogen-free planting materials, monitoring of vectors, suppression of psyllid populations by application of systemic and contact insecticides in the groves, and timely removal of infected trees (26).

Epidemiological models of HLB spread were developed based on symptoms and assumed a linear relationship between infection and symptom expression because the actual timing of HLB infection cannot be determined (21,26). Under greenhouse conditions the incubation period from grafting to development of HLB symptoms is 3-12 months (46).

However, for large trees in a field situation, the incubation period may be much longer, up to five or more years (47,52). The latent period for HLB, the interval between when a plant is infected by a psyllid and the time when that plant can serve as a source of spread by other psyllids, can vary.

The recent rapid spread of HLB in Brazil and Florida (18,28,54) and its impact on these two major citrus industries in a short span of time has led to increased concern amongst growers, regulators and researchers. An analysis of the situation in Pakistan may be useful in understanding the dynamics of citrus HLB in a situation where citrus, the psyllid vector and the pathogen may have coexisted for more than a century. In Pakistan, the disease does not appear to be as severe as reported from southern Florida or the HLB affected area of São Paulo State, Brazil. The long term effects of HLB on the citrus industry in Pakistan seem to be different compared to regions in the western hemisphere where HLB has been introduced recently. A detailed study of the pathosystem from Pakistan would also be of special interest to the United
States of America (USA) since both the Punjab region of Pakistan and many citrus growing regions of the USA have very similar agro-climatic conditions, though only the Punjab region has a monsoon season. Using a climate modeling program, CLIMEX, Hoddle has reported a 70% climate match between citrus production areas of California and the Punjab region of Pakistan (34).

The objectives of the present study were (1) to confirm the presence of the HLB-associated bacterium from both citrus and psyllids in Pakistan using molecular methods, (2) to conduct preliminary sequence analyses and determine if the bacterium is similar to the strain of Las reported from the USA, (3) to assess seasonal variation in psyllid populations, as such information may be useful in devising ways to mitigate psyllid populations by strategically timed spray schedules, and (4) to develop information on the prevalence of Las in psyllids during different seasons which would be beneficial for disease management in Pakistan. The findings are also of interest to California, Texas and Arizona, in the United States, where similar climates occur, the Asian citrus psyllid is becoming established, HLB infected trees are being found, and there is a likelihood that HLB may prevail (42,43).

MATERIALS AND METHODS

Population dynamics of *D. citri*. The insect population study was carried out from April, 2008 to March, 2009. Surveys of *D. citri* populations were conducted in four tehsils (an administrative subdivision): Bhalwal, Kotmomen, Sargodha, and Sillanwali in Sargodha District (Figure 1; regions marked 2, 3, 5, and 7, respectively). A total of 12 orchards were surveyed – three for each tehsil. Monitoring of the populations was carried out using a split plot experimental technique under a randomized complete block design. In each tehsil, three citrus orchards with a
minimum orchard to orchard distance of 5 kilometers were chosen for the study. Five trees were
selected in each orchard in a diagonal pattern with a tree to tree distance of about 50 meters. Five
yellow sticky traps (10×15 cm) were placed on each tree for monitoring the insect populations
(30,32). The yellow sticky traps were hung at 1.5 to 2 meter heights directly on the canopy of
each tree; one trap was placed in each cardinal quadrant and the fifth was positioned in the center
of the tree. The yellow sticky traps were replaced weekly, and the number of psyllid adults in
each trap was recorded. The data collected for 45 weeks was analyzed using SPSS version 19.0
(IBM). The tehsils, orchards, tree quadrants including the center of the tree served as split plots,
complete blocks, and treatments respectively.

Collection of D. citri Samples. Live psyllids were collected using an aspirator from 43 orchards
in 11 different citrus growing regions of Sargodha District (Figure 1). A total of 304 psyllid
samples were collected from April 2009 to July 2010 from individual trees and stored in 95%
ethanol at -20°C at the Institute of Horticultural Sciences, University of Agriculture Faisalabad,
and shipped to USDA ARS, Riverside, CA for qPCR analysis. Each psyllid sample consisted of
2 to 350 adult psyllids collected from a single tree in a specific location. Most samples had about
50 psyllids. A total of 7573 psyllids were analyzed in this study (Tables 1, 2, 3).

Collection of plant samples. Trees showing fruit color inversion, lopsidedness, yellow shoots,
and upright branching pattern were selected for leaf sample collection. Fully expanded and
hardened symptomatic leaves with asymmetric leaf mottle were collected from trees of four
varieties of citrus (‘Kinnow’ mandarin, ‘Fewtrell’s early’ mandarin, ‘Mosambi’ sweet orange
[Citrus sinensis (L.) Osbeck] and grapefruit [C. paradisi Macfad.]) obtained from 43 orchards in
eight citrus growing areas (Figure 1). For each tree sampled, at least 50 symptomatic leaves were
collected, wrapped in aluminum foil, labeled, and transferred to a cool box containing dry ice
and later stored at -80°C at the Institute of Horticultural Sciences, University of Agriculture Faisalabad, until used for DNA extraction. A total of 207 plant samples were collected from 8 regions during spring (March-May) and summer (June-August).

DNA extraction from psyllids. A total of about 2321 psyllid DNA extractions were prepared using multiple (up to 5) psyllid nymphs or adults. MagAttract® 96 DNA plant core kit (Qiagen) was used for insect DNA extraction according to the manufacturer’s protocol, with some modifications. Briefly, the psyllids were air-dried for 10 min, transferred to a 1 ml 96 deep-well, round bottom microplate with each well containing 10 zirconium beads of 2.5 mm diameter. After adding RLT® (Qiagen) extraction buffer (300 µl/well), the plate was covered using capcluster mat (USA Scientific) and homogenized twice for three minutes using a Mini beadbeater (Biospec). The microplate was then processed using the MagAttract® protocol as suggested by the manufacturer. The final DNA eluates (100 µl each) were transferred to a new 96 well round bottom microplate which was sealed, labeled, and stored at -20°C until further use. To calculate the exact percentage of Las positive psyllids from different regions and collected at different time periods, an additional 1357 psyllids were also tested individually (one psyllid per extraction) using the method described above.

Plant DNA extraction. About 0.5 gram of leaf petiole tissue was extracted using CTAB (cetyl trimethyl ammonium bromide) in a laboratory located in Pakistan as previously described (23). Briefly, finely chopped petiole tissue was pulverized in liquid nitrogen using a mortar and pestle and added to 5 ml of hot (65°C) CTAB reagent (containing 2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl, pH 8.0 with 1% polyvinyl pyrrolidone) and emulsified. The emulsion was added to an equal volume of chloroform/isoamyl alcohol (24:1), mixed well, incubated at 65°C for 30 minutes followed by centrifugation and ethanol precipitation. The DNA extracts
were stored at -20°C and shipped in ethanol to USDA ARS, Riverside, CA for molecular studies. At the USDA ARS lab, the DNA was pelleted, washed with cold 70% ethanol, air dried and resuspended in 200 ul 1xTE buffer, pH 8, and used for molecular analyses.

**Multiplex real-time qPCR.** A TaqMan-based real time qPCR assay was used for detection of Las in DNA extracts from psyllids (47). The quality of the DNA in the sample was evaluated by amplification of a fragment of the psyllid gene, Wingless. For DNA extracts from plants, a TaqMan-based real time qPCR assay for amplification of 16S rDNA fragment of Las was conducted (45). The mitochondrial gene, cytochrome oxidase (COX), was used as an internal control. The qPCR assays were performed using a Stratagene Mx3005P qPCR machine and cycle threshold (Ct) values were determined using the Stratagene software. Samples with a Ct value of 34 or less for Las were considered as positive for Las. All qPCR reactions were run simultaneously with many negative controls that consisted of sample extractions without psyllid or plant samples. Using rigorous controls, we feel confident that a Ct value of 34 can be considered as positive for Las.

**Conventional PCR, cloning and sequencing.** Representative qPCR positive plant and psyllid DNA extracts obtained from different regions of Pakistan (with Ct values < 27 for the 16S region) were used to amplify a 1.17 Kb region using 16S rDNA primers, OI1 and OI2c (40). The qPCR positive plant samples were also used for PCR amplification of six other Las genomic regions and the PCR products were cloned in pCR-4 TOPO® (Life Technologies). The clones were subjected to Sanger sequencing using vector-based primers. The genomic regions targeted were: partial ABC transporter gene with a part of intergenic region, fumarate hydrase, FLP/FAP pilin component with intergenic region upstream of this gene, prophage antirepressor region along with part of a gene coding for a hypothetical protein, phage DNA polymerase, and ribonucleotide diphosphate reductase subunit beta. Table 4
lists the genes/regions targeted and primers used for the PCR amplification; the nucleotide
numbering is based on the genomic sequence of Las (24). Sequences obtained were compared
with ‘Candidatus Liberibacter asiaticus’ psy62 strain (24) and unique sequences were deposited
in Genbank. Alignments of sequences were done using Clustal and GeneDoc programs (50,55).

**Weather information.** For the Sargodha region of Pakistan, weather data for 2000-2012 was
obtained from the District Agriculture office, Sargodha, and the Climate Data Processing Centre,
Pakistan Meteorological Dept., Karachi. For regions in the USA, similar data for 1982-2012 was
accessed from this website: http://www.almanac.com/weather/history.

**RESULTS**

**Population dynamics of D. citri.** Using yellow sticky traps placed at different quadrants of
trees, we assessed insect populations in selected citrus orchards during April 2008-March 2009
by recording numbers of adult psyllids. Cardinal trapping patterns remained unchanged among
the different survey areas. Statistical analysis showed temporal and spatial variability in the mean
number of adults trapped on yellow sticky cards. The highest recorded number of adults per trap
per week was during November (33.2 ± 1.06) in the south quadrant of the tree and the lowest
number was during December (0.9 ± 0.17) in the center of the tree (Table 5). Populations
decreased significantly during the winter months of January and February. In the summer months
of June, July and August, when the average maximum temperatures ranged from 35-40°C
(reaching a maximum of 52°C on certain days), more adults were collected from the traps placed
in the center of the tree. During the first population peak period in spring (April-May), a higher
number of psyllids were trapped in northern and central sectors of the tree in comparison to other
tree quadrants but during the second population peak period in autumn (October-November), the
southern and western quadrants had higher numbers. During May (end of spring season), the highest mean number of adults were trapped in the northern quadrants (14.4 ± 1.93) and the central part of the trees (12.4 ± 1.51), a trend that prevailed until the end of summer (August).

After September, east, south and west sides of the trees had significantly higher numbers of adults and this pattern continued until onset of winter (December). During autumn (November), we recorded the highest trapping rate in the southern quadrant (33.20 ± 1.06) followed by the western (28.80 ± 1.0) and eastern (27.90 ± 0.98) quadrants (Figure 2).

Psyllid population counts were recorded weekly from March 2008- April 2009 from four regions in the citrus belt – Sillanwali, Sargodha, Bhalwal and Kot Momen (Figure 3 A-D, corresponding to map regions 6, 5, 2, and 3 in Figure 1). During the entire survey period, two distinct population peaks were observed (Figure 3 A-D); the first peak was during spring from mid-April to mid-May and the second larger peak was in autumn from mid-September to mid-November. The psyllid populations dwindled during two periods, one in winter during January-February and the second lean period was during the hot summer months of June-August (average psyllids per trap ranging from 0-4/trap; Table 5), coinciding with the peak monsoon season with about 68 mm, 96 mm and 149 mm of rainfall in June, July and August of 2008 respectively. Data analysis of weather patterns for 12 years shows a similar trend (Figure 4).

Psyllid population studies started by the end of March 2008, three weeks after the start of the spring flush, when conditions were ideal for insect proliferation; mild temperatures, low rainfall and approximately 80% relative humidity (data not shown; Figure 4 A and B correspond to 12 year averages). During the last week of February (late winter) and 1st week of March (early spring), light to medium showers (resulting in tender flush) and warmer night temperatures of around 15°C were observed and an increase of psyllid populations was recorded by the end of
March (Figure 3A-D). During the population peak in April (mid spring), for the period of this study, the average rainfall was 88 mm, average relative humidity was about 73% (range 44-95%), the average maximum temperature was 33°C (maximum 42°C), and the average minimum temperature was 19°C (minimum 14°C). The number of adult psyllids trapped on yellow sticky traps increased and peaked by the first week of May (Figure 3 panels A, B and C). Towards the end of May (with the onset of summer), the average maximum temperatures reached 39°C (range 29-46°C). A low trapping rate was recorded during July and August (summer) after the onset of the monsoon season in June. Figure 4 B shows rainfall and relative humidity information for the Sargodha region of Punjab province. The citrus plants are irrigated in Pakistan and produce flushes throughout the year except in the winter (December-middle of February). The two major flushing periods were in spring (March-middle of May) and autumn (September-November). Very little flushing was observed during summer months.

There was a second period of rapid increase in D. citri populations from mid-September to mid-November (autumn) after the cessation of the summer monsoon and return of milder temperatures. During the second population peak period of October-November (Figure 3), for the specific period of this study (Apr 2008- March 2009), the maximum daily temperatures averaged 32°C (range 24-39°C) and minimum daily temperatures averaged 18°C (range 9-26°C). Rainfall was low in October (3-11mm) and very low in November (0-0.7 mm) with average relative humidity ranging from 79-86% (49-95% for brief intervals). During the second larger population peak in autumn, the low rainfall, moderate temperatures, and high relative humidity supported a large population increase. There was a steep decline in the number of adults trapped by the end of November, dropping to the lowest recorded level by late December when tender flush was not available and minimum temperatures are closer to 0°C. Either very few or no adult psyllids were
observed on yellow sticky traps from the last week of December to the end of February, when
new spring flushes started to emerge. During January, the average rainfall was 14 mm, relative
humidity was about 90% (range 81-94%), average maximum temperature was 22°C (maximum
30°C), and the average minimum temperature was 8°C (minimum 5°C). The meteorological data
described in this section is for the period when population studies were conducted (April 2008-
March 2009). The average rainfall, relative humidity, maximum and minimum temperatures
recorded for 12 years is shown in Figure 4.

Amongst different regions surveyed, the highest recorded number of psyllids per week from five
trees in a single orchard (25 yellow sticky traps) was over 2600 from Bhalwal region. In other
surveyed regions the captured number of psyllids ranged from 900 in Sillanwali to about 500-
700 in Sargodha and Kotmomen (Figure 3 A-D). It should be noted that data collected from
yellow sticky traps may not represent the psyllid population data accurately since other
environmental factors may influence the number of insects trapped (30).

**Detection of Las in Psyllids.** A total of 305 psyllid samples collected from 43 orchards of 11
citrus growing areas (Figure 1) from 4 districts of Punjab province were tested for the presence
of HLB-associated Las. These samples were analyzed by conducting 2321 multiple psyllid
extractions (2-5 psyllids/extraction) representing a total of 6216 psyllids.

During the months of February-May, 59 out of 139 psyllid samples tested (in 1766
extractions representing 3877 psyllids) were positive (42%) for Las. During summer months of
June and July of 2009 and 2010, 4 out of 54 samples were positive (7%) for Las. 674 psyllids
were analyzed by conducting 181 extractions during this period. Analysis of autumn population
peak from 2009 November showed 8 out of 111 samples positive (7%) for Las. A total of 1665
psyllids were tested by conducting 380 extractions. Monthly results of psyllid testing are shown in Table 1.

The percentage of Las positive samples varied in the different areas surveyed. We tested 106 to 1673 psyllids from each of the eleven regions in multiple psyllid extractions. The percentage of Las positive samples ranged from 9-50. No positive samples were found in three regions, Gojra Talwandi, Lalian and Shahpur from which the psyllid samples were collected only during June or November. The tests indicated widespread presence of Las-positive psyllids in the citrus belt of Pakistan (Table 2) especially during the February to May (spring) period.

A more precise estimate of the percent positive psyllids was obtained by analysis of single psyllids. A total of 1357 psyllids were subjected to single psyllid extractions and tested. A total of 19% positives were obtained: 7.5% in February (n = 40), 12% in March (n = 100), 25% in April (n = 695), 13% in May (n = 496) and 0% in June, July and November (n = 113) (Table 3).

**Detection of Las in Citrus.** Las was detected in 52 plants (n=207) from all areas studied (Figure 5). However, more positives were detected in summer (32%; n=110) than spring (18%; n=97). All four citrus varieties tested, including the two commercial varieties, ‘Mosambi’ and ‘Kinnow’ were found to carry Las. We obtained Ct values as low as 23 (for 16S region of Las in qPCR analysis) from positive plants sampled in June-August when very few Las-positive psyllids were found. This result indicates that during months that are not optimal for detection of Las from the psyllid, the bacterium is detectable from the leaf tissue.

When psyllids are used for testing, March-May (spring) may be the best times to detect HLB associated Las. During the hot summer months of June-July, immediately after the beginning of the monsoon rains, or during the psyllid population peak in autumn, psyllids were
mostly negative for Las. With plants, Las positives could be found in spring as well as in summer, although the relative proportions of live and dead bacteria are not known (Figure 6; additional data not shown). During spring, we found 18% of plant samples (n=97) and 38% of psyllid samples (n=126) to be Las positive. In summer, 32% of plant samples (n=110) and 0.07% of psyllid samples (n=165) tested positive for Las (Figure 6).

**Sequencing of genomic regions of Las isolates from Pakistan.** To confirm the presence of Las in samples by a different method in addition to qPCR, and to compare sequences of the Pakistan Las strain with the sequence of Las psy62 described from Florida (24), we conducted PCR amplifications of selected genomic regions, cloned, sequenced and aligned these sequences of the Pakistan strain of Las to the Florida strain psy62 genomic sequences. Table 4 shows the seven genomic regions PCR amplified from *D. citri* and plant DNA obtained from various regions of Pakistan. In five of the seven regions analyzed, the sequences of Pakistan strain of Las was 99-100% identical to the Florida strain. Interestingly, in two regions identified as having bacteriophage related sequences, there was 10-15% sequence variability compared to Las strain psy62 from Florida and also when compared to samples obtained from Shamsabad, Lalian, Kot Momen and Sargodha regions of Pakistan. All unique sequences were deposited in GenBank (Table 4 shows the GenBank accession numbers). Supplementary Figure 1 shows nucleotide alignment of the phage region that shows differences between the Pakistan isolates and the Florida strain, psy62.

**DISCUSSION**

The citrus industry in Pakistan has been hampered by citrus decline for over a century. Reports of citrus diseases similar to HLB, described as citrus dieback, date back to the 18th
century (reported by Roghoji Bhonsale, cited by Capoor; 13). In a 1912 report of the insects belonging to the Family Psyllidae collected from various regions of India, Crawford lists 14 psyllid genera including *D. citri* (as *Euphalerus citri* (Kuwayama)) (19,20,48). The psyllids were reported to be present in “considerable numbers” on citrus trees in 1909 (19); the diagrams published in Crawford’s paper show wing morphology identical to *D. citri* that is now common in Florida while the male and female genitalia are slightly different from *D. citri*. Since the area described is the center of origin for the *Diaphorina* species that colonize Rutaceae (27,37), there might be several closely related species (Halbert, personal communication). There were reports of serious attack of citrus trees by *D. citri* in 1916 in the Sargodha region of Pakistan resulting in substantial financial losses (38). Husain and Nath (38) were the first to report damage in citrus infested with *D. citri* and described symptoms that match the HLB symptoms described today. They further hypothesized that the psyllids inject some poison to cause symptoms. Although several factors were implicated in citrus dieback (7), it appears probable that the presence of psyllids as early as 1909 (19), the citrus dieback symptoms and the resulting loss of yield associated with it known to exist at the time and the reports of yield loss in citrus trees infested with *D. citri* (38) indicate that HLB was most likely the major factor responsible for citrus decline in the Indian sub-continent for at least 100 years. It is probable that HLB associated with *D. citri* (then known as citrus dieback) was present in the Indian sub-continent before moving to China (36). Husain and Nath (38) conducted their studies in Sargodha and Faisalabad (then known as Lyallpur) and surrounding regions where the present study was also carried out (Figure 1).

In Pakistan, *D. citri* is widespread and has co-existed with citrus for over a century. The disease has been endemic in the Indian subcontinent for a long time (7). Since maximum
diversity of the genus *Diaphorina* is known to exist in the Indian subcontinent, it is presumed to be the place of origin of the Asian citrus psyllid (10, 27, 37). In the western hemisphere, where HLB has been introduced recently, the disease progression has been rapid. In a large citrus orchard in south Florida, the logistic rate of disease increase was estimated to be in the range of 0.002 to 0.39 in a period of 10 months (26). Active HLB mitigation efforts are not known in Pakistan. At this rate of disease spread the citrus industry could not have survived with the psyllid and possibly with *Ca. L. asiaticus* for at least 100 years. Citrus is still grown commercially in many regions of the Indian subcontinent, including the Punjab area of Pakistan, indicating that the disease is not as severe as presently seen in Brazil or Florida, and that a delicate balance between the host, vector, the environment and the disease may exist.

The liberibacters associated with citrus HLB differ in their sensitivity to high temperatures (12). Many recent greenhouse and laboratory experiments conducted by scientists in Brazil and Florida indicate the temperature sensitivity of the HLB-associated psyllid and the implication is that, in very hot climates, HLB may not become a serious concern. Lopes et al. (46) conducted experiments using citrus cultivars infected with *Ca. L. asiaticus* and *Ca. L. americanus* by incubating potted citrus trees in growth chambers maintained at different temperatures. *Ca. L. americanus* was sensitive to high temperatures and was barely detectable (titer of $10^1$ cells per gram of leaf midrib) from infected plants incubated at 35°C. *Ca. L. asiaticus* was considered “heat tolerant” since plants exposed to 35°C had high titers ($10^7$ cells per gram of leaf midrib) of the bacterium. However, when incubated at 38°C for six hours per day, over a period of 90 days, *Ca. L. asiaticus* titers reduced from $10^7$ cells to about $10^3$ cells per gram of leaf midrib. It was concluded that the heat tolerant *Ca. L. asiaticus* can withstand up to a maximum of 35°C (46). In experiments conducted in Florida under greenhouse situations,
Hall et al. (33) observed a linear increase in the number of eggs laid by the psyllid between 17 and 32°C. Oviposition was significantly reduced above this temperature and at 41°C, the mortality of *D. citri* was very high.

The Asian citrus psyllid can survive temperatures as high as 45°C, as observed by Aubert in Saudi Arabia (10,33). In Pakistan, the average day time summer temperatures are 38°C to 40°C from May-July, often reaching 45°C and occasionally exceeding 50°C (Figure 4). Although the populations decline, the psyllid survives (Figure 3). In the present study, the psyllid testing analysis indicated that, during relatively hot periods, the HLB-associated bacterium was not detectable in *D. citri* (Table 1) but the bacterium was easily detectable from plants (Figure 6; Ct values of 23 to 32 for 16S rDNA region). Las detected from plants during hot weather may not be transmissible since the psyllids collected during both summer and the following autumn showed a very low percentage of Las positives. In addition, the qPCR assay used does not differentiate between living and dead bacteria. The bacteria may also inhabit the cooler regions of the plant such as the inner canopy and roots (58).

Multiple environmental factors in Pakistan may determine the survival of both the vector and the bacterium (53). Average maximum temperatures in citrus growing areas of California, Texas and Arizona reach 35°C to 40°C for about three months in a year (Figure 7), often reaching 45°C or exceeding 50°C (in Arizona). Observations recorded by Lopes et al. (46) suggest that the effect of HLB on citrus plants may be relatively milder in these regions because of hot summer temperatures. However, our observations in Pakistan suggest that the effect of temperature on the vector and the bacteria is complex and may be mitigated by other environmental factors. Beattie et al. observed that high saturation deficits favor *D. citri* populations at temperatures above 40°C (personal communication). When relative humidity is
not high and host plant leaves remain turgid, psyllids may survive due to evaporative cooling
(10).

The possibility of subjecting HLB positive citrus plants to high temperatures for
thermotherapy is being investigated by several researchers (35). While this is a promising
approach to mitigate HLB in a controlled environment, the utility of such an approach for field
trees may be limited. Our results show that the crop of psyllids during November (autumn) do
not carry Las probably because maximum temperatures reaching up to 50°C or higher during the
summer months are detrimental to the bacteria in the insect vector. Although we were able to
detect the pathogen from mature leaves during the hot summer months, it is probable that most
bacteria in the plant tissue are either dead or non-transmissible since the psyllid population in the
autumn season (September-November) is mostly Las negative (Table 1). In Florida, the highest
percentage of Las positive psyllids are usually found in October-November (47), in direct
contrast to our findings in Pakistan. We hypothesize that higher summer temperatures in Pakistan
may be a major factor contributing to this difference, probably resulting in either dead or non-
transmissible bacteria in shoots. Natural thermotherapy may occur during hot seasons in plants,
but when temperatures are favorable, bacteria in citrus roots may move to the tender flush and, as
the bacterial titer builds up, the psyllids are able to reacquire the pathogen. This phenomenon
may be leading to the natural mitigation of HLB under field conditions and might have
contributed to the long term survival of citrus industry in Pakistan.

We compared the average, minimum and maximum temperatures recorded for the citrus-
growing regions of United States (over a 30 year period) with Sargodha, Pakistan (over a 12
year period; Figure 7). Maximum temperatures of 50°C and above is probably a major factor
responsible for eliminating Las from the psyllids and resulting in presumably dead and/or non-
transmissible Las in citrus plants in Pakistan. Among citrus growing regions of the US, such high
temperatures are recorded only from Arizona. The maximum summer temperatures recorded in
California are about 6-7°C lower than Pakistan and may or may not be sufficient to eliminate Las
from the psyllids. Beattie et al. have identified other environmental factors in addition to
temperature as having a role in the presumed thermotherapy phenomenon. Humidity and
saturation deficits may also influence the microclimate and affect the body temperature of the
psyllids and citrus leaves (10). All these factors may determine the Las titers in the psyllid vector
and in the plant canopy after summer months.

In Pakistan, the winter temperatures during December and January (average minimum
temperatures of 5-7°C with a minimum of 0°C) resulted in a low population peak for the psyllid.
Winter temperatures in all the citrus growing regions of the US are much lower than the
temperatures recorded in Sargodha and may result in a population decline during winter months.
During the two population peaks in April and November, the maximum temperatures in Pakistan
and the citrus growing regions of the US are comparable (5-6°C lower in California during
November). The minimum temperatures are much lower (10-12°C) in California than in
Pakistan. The population peaks will also be determined by many other factors like rainfall,
presence of tender flush, humidity, etc. (9).

The psyllid population dynamics varies in different climatic situations. In our study we
collected psyllids from four regions – Sillanwali, Sargodha, Bhalwal and Kotmomen and
observed certain variations in psyllid population peaks. It appears that a population peak during
April (spring) is a common occurrence in Pakistan. During April, there was a population peak in
three of the four regions (not in Kot Momen orchard). Since this was not a controlled study,
efforts were not made to instruct the local citrus growers to follow any specific psyllid control
procedures. We were interested in assessing the current disease status in Pakistan and obtaining information that would lead to a better understanding of the HLB situation.

The local psyllid population dynamics will vary depending on many factors, including availability of tender flush (31,53). Presence of citrus relatives that provide tender flush throughout the year would have a positive effect of psyllid populations (58). Rainfall pattern will have an effect on the presence of tender flush, direct impact on survival of psyllid eggs and nymphs, and, on leaf temperatures (53). Temperature, humidity, nutritional status of the plants, presence of natural enemies and local orchard management practices are known to influence psyllid population dynamics (53). In a 3-year study at Haryana, India, Lakra et al. (44) have identified key psyllid mortality factors as: maximum daily temperatures of 45°C or above coupled with low relative humidity (<40%) during May-June, low minimum temperatures (below 5°C) during December-February and heavy rains (more than 300 mm) during July (44,59). The population dynamics of psyllids in recently invaded areas should be studied in detail and correlated with local meteorological data to plan spray schedules that reduce psyllid numbers and mitigate the disease.

We detected ‘Ca. L. asiaticus’ in about 22% of a total of 305 psyllid samples by qPCR using multiple psyllid extractions. Psyllids collected from most areas in this study were positive for ‘Ca. L. asiaticus’ (Table 2) indicating the widespread occurrence of the pathogen. In two regions, Mandi Bahuddin and Lalian, very low numbers of Las positive psyllids were documented. At these locations, psyllids were collected during summer months when positive psyllids are known to be in low numbers (Table 1). Pathogen detection was higher in psyllid samples collected during March- May (spring) during two successive years (Table 1), compared to other seasons. Based on our data, in Pakistan, it may be advisable to do psyllid testing during
March–May since many positive psyllids were found during this period. Although November is the peak period for psyllid populations, we could not detect Las from psyllids during autumn. Apparently the nymphs that develop into the November adult population did not acquire Las from citrus.

In São Paulo State, Belasque et al. have shown that, when active disease management efforts are not practiced, percentage of HLB affected trees increased from 2% to about 16% within a single year (11). In many parts of Pakistan, HLB has been in existence for almost a century (38). These orchards were not aggressively treated to control the psyllid nor were the diseased trees removed. Presumably, opportunities for disease spread and establishment are optimal. However, diseased trees are still yielding fruit, albeit at much reduced levels. The dynamics of disease development in Pakistan are different from Brazil or the USA where ‘Ca. L. asiaticus’ is a recently introduced pathogen. In Pakistan, the plant host, insect vector and the pathogen have co-existed for over a century and citrus is still commercially grown. Many factors could contribute to this scenario, including increased tolerance in citrus hosts, differences in the Las genotypes or, most importantly, environmental factors. The absence of Las in psyllids during autumn and, presumably, presence of mostly dead Las in citrus leaves after harsh summer temperatures might contribute to maintenance of a balance between the host, pathogen and the disease.

To test if the Las strain prevalent in Pakistan is different from the Las psy62 strain described from Florida, we have conducted limited sequence analysis of seven genomic regions of ‘Ca. L. asiaticus’ obtained from citrus plants from different regions of the Sargodha area. The sequences generated from the ‘Ca. L. asiaticus’ strain from Pakistan (Table 4) are about 99 percent similar to the Florida strain in 5 genomic regions. However, the two phage regions
studied showed a significant amount of variability. Further characterization of the variable regions in the different populations of ‘Ca. L. asiaticus’ may be useful in understanding the disease dynamics of the citrus/HLB-pathosystem in Pakistan.

Management of HLB in Pakistan is challenging due to the absence of nursery regulations mandating use of disease free planting material, widespread occurrence of the psyllid vector and the presence of HLB for a long period of time. The practice of plant propagation by air-layering from HLB infected trees has lead to the nearly complete disappearance of sweet lime (Citrus limettiioides Tan.) plantations in Pakistan. Even though HLB has been known from the Indian sub-continent for about 100 years (26), there has not been a detailed study to detect and characterize the HLB-associated pathogen from Pakistan. The present study is an effort to assess the actual status of HLB in Pakistan based on sensitive detection methods and molecular characterization of ‘Ca. L. asiaticus’ isolates from both the citrus plants and the insect vector, along with the analysis of vector preponderance data. An understanding of the psyllid population dynamics and the disease situation under these conditions may be of immense value to the Pakistan citrus industry. Disease mitigation is difficult in older orchards since the usual management practices may not be adequate to cope with the damage. However, planting of new citrus orchards using disease-free plants raised in protected nurseries and area-wide management of the vector should be done as is being practiced in parts of Florida, Texas, and Brazil. Establishment of facilities for molecular detection would be critical for proper identification of HLB positive psyllids and plants. Local monitoring of the psyllid populations (as shown in Figure 3) will be helpful in identifying critical periods when insect control is logistically feasible and financially affordable. Our study shows that D. citri is very well established throughout the Punjab province of Pakistan. The psyllid population is low during hot summer months (June to
August) and also during cold months (January-February), and insecticide sprays during these two times may be effective in controlling psyllid populations. Additional sprays may be applied as needed. In Florida, insecticide sprays for management of psyllids are recommended during winter when the population is low (dormant sprays; 51).

HLB has not been adequately controlled anywhere in the world. It is an invasive disease that is known to spread rapidly in new areas and cause immense crop losses. However, in Pakistan, the pathogen, its insect host and the plant host have co-existed for a very long time (38). Although damage is evident and the yield of citrus much lower compared to other citrus growing regions of the world, continued presence of the pathogen and the vector have not completely destroyed the citrus industry. Further studies of this pathosystem in Pakistan would be useful in developing management techniques in places with similar climatic conditions like California and Texas in the US where HLB has been recently introduced.

**Acknowledgements**

Excellent technical help received from Lupe Heldoorn and Esteban Rodriguez is acknowledged. Laboratory support for DNA extractions in Faisalabad, Pakistan was kindly provided by Dr. Ehsan Iqbal, University of Agriculture, Faisalabad. We appreciate the expert advice provided by Dr. Susan Halbert in proper identification of psyllid diagrams reported from India in a 1912 publication. This research was made possible by support from the Pakistan – U.S. Science and Technology Cooperation Program, California Citrus Research Board and the Higher Education Commission of Pakistan.
LITERATURE CITED


Table 1. Psyllid testing at different time points in four regions of Pakistan.

<table>
<thead>
<tr>
<th>Month of Collection</th>
<th>Samples No.</th>
<th>Las positive</th>
<th>Percent positive</th>
<th>Extractions No.</th>
<th>Las positive</th>
<th>Percent positive</th>
<th>Total psyllids analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feb</td>
<td>13</td>
<td>11</td>
<td>85</td>
<td>72</td>
<td>14</td>
<td>19</td>
<td>202</td>
</tr>
<tr>
<td>Mar</td>
<td>56</td>
<td>8</td>
<td>14</td>
<td>315</td>
<td>22</td>
<td>7</td>
<td>1178</td>
</tr>
<tr>
<td>Apr</td>
<td>34</td>
<td>24</td>
<td>71</td>
<td>872</td>
<td>230</td>
<td>45</td>
<td>1584</td>
</tr>
<tr>
<td>May</td>
<td>36</td>
<td>16</td>
<td>44</td>
<td>507</td>
<td>88</td>
<td>17</td>
<td>913</td>
</tr>
<tr>
<td>Jun</td>
<td>11</td>
<td>1</td>
<td>9</td>
<td>42</td>
<td>1</td>
<td>2</td>
<td>142</td>
</tr>
<tr>
<td>Jul</td>
<td>43</td>
<td>3</td>
<td>7</td>
<td>139</td>
<td>3</td>
<td>6</td>
<td>532</td>
</tr>
<tr>
<td>Nov</td>
<td>111</td>
<td>8</td>
<td>7</td>
<td>380</td>
<td>8</td>
<td>2</td>
<td>1665</td>
</tr>
</tbody>
</table>

*Extractions were made from 5 psyllids.*
Table 2. Detection of Las in psyllids collected from eleven regions of Pakistan.

<table>
<thead>
<tr>
<th>Region</th>
<th>Samples</th>
<th>Extractions</th>
<th>Total no. of psyllids analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Las +</td>
<td>Percent Positive</td>
</tr>
<tr>
<td>Bhalwal</td>
<td>51</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>Faisalabad</td>
<td>93</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Gojra Talwandi&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Jhang, Shamasabad</td>
<td>27</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td>Kotmomen</td>
<td>15</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Lalian&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Mandi Bahauddin</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sahiwal</td>
<td>6</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Sargodha</td>
<td>32</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Shahpur&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Toba Tek Singh</td>
<td>30</td>
<td>13</td>
<td>43</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>305</td>
<td>67</td>
<td>22</td>
</tr>
</tbody>
</table>

<sup>a</sup>Extractions were conducted using mostly 2-5 psyllids per extraction.

<sup>b</sup>Collected during November when percentage of Las positive psyllids is very low in Pakistan.

<sup>c</sup>Collected in June when percentage of Las positive psyllids is very low in Pakistan.
Table 3. Single psyllid extractions tested for the presence of Las.

<table>
<thead>
<tr>
<th>No.</th>
<th>Area</th>
<th>Date of Collection</th>
<th>Psyllids tested</th>
<th>Las +</th>
<th>Percent positives</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Jhang, Shamsabad</td>
<td>11-Feb-2010</td>
<td>7</td>
<td>0</td>
<td>0</td>
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<tr>
<td>2</td>
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<td>11-Feb-2010</td>
<td>7</td>
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<td>14</td>
</tr>
<tr>
<td>3</td>
<td>Jhang, Shamsabad</td>
<td>11-Feb-2010</td>
<td>8</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Jhang, Shamsabad</td>
<td>11-Feb-2010</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Jhang, Shamsabad</td>
<td>11-Feb-2010</td>
<td>9</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>Bhalwal</td>
<td>5-Mar-2010</td>
<td>39</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Bhalwal</td>
<td>26-Mar-2010</td>
<td>4</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td>8</td>
<td>Bhalwal</td>
<td>26-Mar-2010</td>
<td>17</td>
<td>9</td>
<td>53</td>
</tr>
<tr>
<td>9</td>
<td>Mandi Bahauddin</td>
<td>22-Mar-2010</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Faisalabad</td>
<td>16-Apr-2009</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>Faisalabad</td>
<td>16-Apr-2009</td>
<td>22</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>Faisalabad</td>
<td>16-Apr-2009</td>
<td>22</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>13</td>
<td>Faisalabad</td>
<td>16-Apr-2009</td>
<td>22</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>14</td>
<td>Toba Tek Singh</td>
<td>2-Apr-2010</td>
<td>69</td>
<td>29</td>
<td>42</td>
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<tr>
<td>15</td>
<td>Toba Tek Singh</td>
<td>2-Apr-2010</td>
<td>82</td>
<td>43</td>
<td>52</td>
</tr>
<tr>
<td>16</td>
<td>Toba Tek Singh</td>
<td>2-Apr-2010</td>
<td>131</td>
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<td>18</td>
<td>Toba Tek Singh</td>
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<td>108</td>
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<td>44</td>
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<tr>
<td>19</td>
<td>Toba Tek Singh</td>
<td>6-Apr-2010</td>
<td>187</td>
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<td>20</td>
<td>Bhalwal</td>
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<td>21</td>
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<td>10</td>
<td>13</td>
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<tr>
<td>22</td>
<td>Bhalwal</td>
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<td>13</td>
<td>16</td>
</tr>
<tr>
<td>23</td>
<td>Kot Momen</td>
<td>7-May-2009</td>
<td>48</td>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td>24</td>
<td>Sahiwal</td>
<td>7-May-2009</td>
<td>41</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>25</td>
<td>Sahiwal</td>
<td>7-May-2009</td>
<td>87</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>26</td>
<td>Shahpur</td>
<td>21-Jun-2010</td>
<td>18</td>
<td>0</td>
<td>0</td>
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<tr>
<td>27</td>
<td>Sargodha</td>
<td>14-Jul-2010</td>
<td>41</td>
<td>0</td>
<td>0</td>
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<tr>
<td>28</td>
<td>Faisalabad</td>
<td>6-Nov-2009</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>29</td>
<td>Faisalabad</td>
<td>27-Nov-2009</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>Lalian</td>
<td>21-Nov-2009</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>1357</td>
<td>253</td>
<td>19</td>
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Table 4. Primers used to amplify genomic regions of Las from positive plant and psyllid samples.

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Sequence</th>
<th>Position&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Putative gene product</th>
<th>Reference</th>
<th>Genbank accession no. (Source)</th>
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<tbody>
<tr>
<td>Cit 205</td>
<td>GCGCGTATGCAATACGAGCGGCA</td>
<td>418261</td>
<td>16S rRNA</td>
<td>49</td>
<td>JQ866401 (Psyllid)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>JQ866408 (Citrus)</td>
</tr>
<tr>
<td>Cit 206</td>
<td>GCCTCGCGACTTCGCAACCCAT</td>
<td>417090</td>
<td>16S rRNA</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Cit 700</td>
<td>TGGAGTGATAAAATTACCACG</td>
<td>19021</td>
<td>ABC transporter protein</td>
<td>This study</td>
<td>JQ866402 (Citrus)</td>
</tr>
<tr>
<td>Cit 701</td>
<td>GCTACTTATTAGTCTCGGAA</td>
<td>19751</td>
<td>Intergenic region downstream of ABC transporter protein</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Cit 702</td>
<td>CTCTTGTATCTACGTGCA</td>
<td>76291</td>
<td>Fumarate hydrase</td>
<td>This study</td>
<td>JQ866403 (Citrus)</td>
</tr>
<tr>
<td>Cit 703</td>
<td>GCAACCCTGTATATGTCTC</td>
<td>76920</td>
<td>Fumarate hydrase</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Cit 706</td>
<td>GCCGCTACTGAATATGT</td>
<td>534654</td>
<td>Intergenic region upstream of Flp/Fap pilin component</td>
<td>This study</td>
<td>JQ866404 (Citrus)</td>
</tr>
<tr>
<td>Cit 707</td>
<td>GTGGTAACGGAAGTGAT</td>
<td>535322</td>
<td>FLP/FAP pilin component</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>Cit 708</td>
<td>GAGGTGTACCTACTCTTCG</td>
<td>2570</td>
<td>Prophage antirepressor</td>
<td>67</td>
<td>JQ866406 (Citrus)</td>
</tr>
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<td></td>
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<td></td>
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<td>JQ866405 (Citrus)</td>
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<tr>
<td>Cit 709</td>
<td>GTATCAAGAGCAGGGTACG</td>
<td>3140</td>
<td>Hypothetical protein</td>
<td>67</td>
<td></td>
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<tr>
<td>Cit 710</td>
<td>CTCCGTATGACTGTACTCGTGC</td>
<td>4172</td>
<td>Phage DNA polymerase</td>
<td>67</td>
<td>JQ866407 (Citrus)</td>
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<td>B 21</td>
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<tr>
<td>B 26</td>
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<td>11466</td>
<td>Ribonucleotide-diphosphate reductase subunit</td>
<td>This study</td>
<td></td>
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<sup>a</sup> Nucleotide position indicates coordinates in Las sequence (GenBank accession no.: CP001677.4) All unique sequences were deposited in GenBank (last column).
Table 5. Mean number of adult psyllids trapped (± standard error) on yellow sticky cards placed at different quadrants in the trees.

<table>
<thead>
<tr>
<th>Sampling Months</th>
<th>East</th>
<th>South</th>
<th>West</th>
<th>North</th>
<th>Center</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>10.05 ± 1.43</td>
<td>9.95 ± 1.36</td>
<td>10.4 ± 1.37</td>
<td>11.31 ± 1.61</td>
<td>8.22 ± 1.06</td>
</tr>
<tr>
<td>May</td>
<td>8.55 ± 1.38</td>
<td>9.71 ± 1.52</td>
<td>9.37 ± 1.41</td>
<td>14.38 ± 1.93</td>
<td>12.44 ± 1.51</td>
</tr>
<tr>
<td>June</td>
<td>0.74 ± 0.095</td>
<td>1.06 ± 0.15</td>
<td>1.23 ± 0.14</td>
<td>1.68 ± 0.21</td>
<td>3.58 ± 0.42</td>
</tr>
<tr>
<td>July</td>
<td>1.12 ± 0.13</td>
<td>0.72 ± 0.10</td>
<td>0.81 ± 0.086</td>
<td>1.5 ± 0.14</td>
<td>2.23 ± 0.21</td>
</tr>
<tr>
<td>August</td>
<td>0.59 ± 0.05</td>
<td>0.3 ± 0.04</td>
<td>0.21 ± 0.03</td>
<td>0.85 ± 0.096</td>
<td>0.7 ± 0.071</td>
</tr>
<tr>
<td>September</td>
<td>4.28 ± 0.22</td>
<td>2.67 ± 0.16</td>
<td>2.3 ± 0.15</td>
<td>3.17 ± 0.17</td>
<td>1.99 ± 0.11</td>
</tr>
<tr>
<td>October</td>
<td>23.66 ± 1.00</td>
<td>21.36 ± 0.95</td>
<td>21.7 ± 0.97</td>
<td>16.1 ± 0.64</td>
<td>7.43 ± 0.25</td>
</tr>
<tr>
<td>November</td>
<td>27.85 ± 0.98</td>
<td>33.18 ± 1.06</td>
<td>28.84 ± 1.00</td>
<td>12.02 ± 0.55</td>
<td>3.65 ± 0.19</td>
</tr>
<tr>
<td>December</td>
<td>5.32 ± 0.46</td>
<td>6.61 ± 0.53</td>
<td>5.04 ± 0.43</td>
<td>1.73 ± 0.15</td>
<td>0.88 ± 0.17</td>
</tr>
<tr>
<td>January</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>February</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>March</td>
<td>2.89 ± 0.17</td>
<td>2.85 ± 0.18</td>
<td>2.68 ± 0.17</td>
<td>1.5 ± 0.10</td>
<td>0.95 ± 0.08</td>
</tr>
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Figure captions:

Figure 1. Map showing citrus growing regions of Sargodha District and surrounding regions in the Punjab Province of Pakistan where citrus orchards were selected for studies on population dynamics of *Diaphorina citri*, and for analysis of psyllids and citrus for the presence of *Candidatus Liberibacter asiaticus* associated with citrus huanglongbing disease. Regions targeted for the study were: Mandi Bahauddin (1), Bhalwal (2), Kotmomen (3), Shahpur (4), Sargodha (5), Sillanwali (6), Lalian (7), Faisalabad (8), Jhang/Shamsabad (9), Gojra (10) and Toba Tek Singh (11).

Figure 2. Mean number of insects (*Diaphorina citri*) trapped on yellow sticky cards (per trap per week) placed at the four sides of the tree and in the center of the canopy in November, 2009 when the psyllid population reached its highest level. Standard error bars are shown.

Figure 3. Population dynamics of *Diaphorina citri* in four different ‘Kinnow’ mandarin orchards located in Sillanwali (A, region 6; refer to Figure 1), Sargodha (B, region 5), Bhalwal (C, region 2) and Kot Momen (D, region 3). Total number of psyllids trapped on 25 yellow sticky traps (10cm X 15 cm) on five trees at each location was recorded. Weekly data for 45 weeks shown (from April 2008 to March 2009).

Figure 4. Weather data from Sargodha, Pakistan. A. Average minimum (white rectangles) and average maximum temperatures (shaded rectangles) from a 12 year dataset (2000-2012) are shown. The temperature range for each time period is indicated by error bars. The line graph shows average number of psyllids captured on yellow sticky traps from the Sargodha region. B. Average rainfall and relative humidity in Sargodha region. Rainfall data in mm (bar graph) and relative humidity (expressed as % relative humidity recorded at 8AM; line graph). Average values for each month are plotted. The maximum and minimum ranges reported are shown by error bars. Weather information was obtained from the district Agriculture office, Sargodha and the Meteorological Department, Karachi, Pakistan.

Figure 5. Analysis of plant samples for the presence of Las. Citrus samples were collected from eight regions in the Pakistan citrus belt are shown on X axis. Percent positive samples for *Candidatus Liberibacter asiaticus* are shown on Y axis. Number of samples tested was: 43 (Bhalwal), 27 (Faisalabad), 24 (Jhang, Shamsabad), 10 (Kot Momen), 44 (Sargodha), 3 (Sillanwali) and 10 (Toba Tek Singh).

Figure 6. Summary of testing of psyllids and citrus during spring and summer for the presence of *Candidatus Liberibacter asiaticus* (Las). During March to May, 126 psyllid samples (3675 psyllids) and 97 plant samples were tested for the presence of Las. During June to August, 165 psyllid samples (674 psyllids) and 110 plant samples were analyzed. Open bars represent psyllid positives and shaded bars represent plant positives.
Figure 7. Comparison of maximum and minimum temperatures of Sargodha, Pakistan with citrus growing regions of USA. Panel A shows maximum temperatures and panel B shows minimum temperatures. Black rectangles represent average temperatures in Sargodha, Pakistan. The range observed is shown by solid black error bars. Red squares indicate the average temperatures in Bakersfield, California. Red dotted lines show the range recorded. Blue X marks show the average temperatures recorded in Brownsville, Texas and the blue dashed lines show the range. Green circles represent average temperatures in Yuma, Arizona and the green solid lines indicate the range. Grey triangles show the average temperatures in Lake Alfred, Florida and grey dotted and dashed lines indicate the range observed. For Sargodha, average of 12 year data (2000-2012) was plotted. For all other regions, 30 year data is plotted (1982-2012).

Supplementary Figure 1. Alignment of sequences obtained from the prophage region of Las from plant samples collected from Sargodha (PX 171), Kot Momen (PX 186), Lalian (PX 212) and Shamsabad (PX 410) areas of Pakistan showing sequence variation when compared to the fully sequenced Las genome (corresponding to nucleotides 2929-3140 of Las genome from Florida, GenBank accession no. CP001677.5) Significant differences are indicated in boxes.
Figure 1.
Figure 2.

Mean number of insects trapped

- East
- South
- West
- North
- Center
Figure 3.
Figure 4.
Figure 5.

Las-positive plant samples (%)

- Bhalwal
- Faisalabad
- Jhang, Shamsabad
- Kot Momen
- Lalian
- Sargodha
- Sillanwali
- Toba Tek Singh

0 10 20 30 40 50
Figure 6.

![Bar chart showing positive samples (%) for Psyllid and Plant over Mar/Apr/May and Jun/Jul/Aug.]
Figure 7.

A

Average maximum temperatures

- Sargodha, Pakistan
- Bakersfield, California
- Brownsville, Texas
- Yuma, Arizona
- Fort Pierce, Florida

B

Average minimum temperatures

Temperature °C

10 Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

0 -10
Supplementary Figure 1.